

**Co-Transplantation of adult neural stem/progenitor cells
together with cells of mesenchymal origin into the
injured spinal cord**



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Summary

The irreversible loss of spinal cord parenchyma including astroglia, oligodendroglia and neurons is one of the key factors responsible for the severe functional impairment in individuals suffering from spinal cord injury. Therefore, adequate cell replacement strategies might be one means to promote structural and functional recovery. Neural stem/progenitor cells (NPC), which have been identified in the adult mammalian nervous system including the spinal cord, represent one promising source to replace scaffold forming astrocytes, remyelinating oligodendrocytes and neurons within the injured spinal cord. Intrinsic neural stem/progenitor cells at and around the lesion site can be stimulated by the application of appropriate molecules to replace lost spinal cord tissue intrinsically (stimulation of endogenous cell replacement). Alternatively, neural stem cells can be isolated from small brain/spinal cord biopsies, propagated *in vitro* and ultimately transplanted into the injured spinal cord (neural stem cell transplantation).

Recently it has been published that mesenchymal stem cells (MSC) secrete a yet unidentified factor, which strongly promotes oligodendroglial differentiation of hippocampus derived adult neural progenitor cells *in vitro* under co-culture conditions, whereas the astrogenic commitment of NPC is inhibited.

Based on these findings, I investigated whether the region of isolation (origin) of NPC will influence the expression pattern of specific differentiation markers after incubation with MSC-conditioned media (MSC-CM). I could show that MSC-derived soluble factors induce the expression of oligodendrocyte markers in NPC *in vitro* regardless of the origin of the NPC. Furthermore, incubation of NPC with conditioned media derived from fibroblasts resulted in an even higher number of cells expressing the oligodendroglial marker MBP at the expense of cells expressing the astroglial marker GFAP. These data and the fact that MSC and fibroblasts share the same mesenchymal origin suggest that MSC-derived soluble factors and fibroblasts-derived soluble factors act via the same signaling pathway.

In the next step, NPC or NPC pre-differentiated towards an oligodendroglial

lineage were co-seeded with MSC onto hippocampal slice cultures. Under CNS-organotypic conditions MSC still promoted an oligodendroglial fate of seeded NPC. While the survival of the seeded NPC was good, the survival of oligodendroglial pre-differentiated NPC was very limited after seeding onto hippocampal slices.

To see if the pro-oligodendrogenic activity of MSC is maintained *in vivo*, NPC or pre-differentiated NPC were co-transplanted with MSC into the intact spinal cord of adult rats. Although the survival of pre-differentiated NPC was very low, a significantly increased oligodendroglial differentiation was observed when compared to NPC co-grafted with MSC.

In subsequent experiments, NPC were co-grafted with MSC or fibroblast into the injured spinal cord. Histological analysis demonstrated that fibroblast as well as MSC containing grafts filled the cystic lesion after SCI and provided a supporting scaffold to sustain adult NPC within the lesion cavity. Interestingly, fibroblasts but not MSC increased the oligodendroglial differentiation of co-grafted NPC in the injured spinal cord. In vitro data demonstrated that BMP2 and BMP4 (bone morphogenic protein 2 and 4), which are strongly up-regulated after spinal cord injury completely counteracted effects of MSC, on oligodendroglial differentiation of NPC. Thus, neutralization of BMPs or BMP signaling might be necessary to enhance oligodendroglial differentiation by MSC *in vivo*.

Moreover, my studies revealed that the transplantation of MSC into the injured spinal cord does not alter the proliferation or survival of endogenous NPC. Rather MSC influence the differentiation of endogenous oligodendroglial progeny as early as three days after SCI and shift the differentiation pattern of NPC towards an oligodendroglial phenotype four weeks after SCI at the expense of astroglial differentiation.

In summary, these studies demonstrate that MSC provide a pro-oligodendrogenic microenvironment for NPC seeded onto hippocampal slices or transplanted into the intact spinal cord. In contrast, MSC do not influence the differentiation of co-transplanted NPC in the acutely injured spinal cord, but profoundly affect the differentiation of endogenous NPC.

For any cell-based therapy to be translated into the clinic appropriate monitoring tools need to be established to visualize morphological changes

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caused by cell transplantation into the injured spinal cord. Magnetic resonance imaging (MRI) represents the gold-standard to non-invasively visualize the spinal cord parenchyma. As a first step to validate cell-therapy induced morphological changes, I performed analysis using a routine clinical 3T MRI-scanner. The referring study demonstrated that a routine clinical 3T MRI-scanner can be used for small animal imaging to noninvasively visualize pathological changes occurring after rat spinal cord injury. Changes in 3T MRI signals correlate with histological, structural and behavioral (locomotor) outcomes after SCI.

Zusammenfassung

Der irreversible Verlust von Rückenmarksgewebe insbesondere Astroglia, Oligodendroglia und Neuronen ist eine der Hauptursachen für schwerwiegende funktionellen Beeinträchtigungen in Individuen, die eine Rückenmarksverletzung erleiden. Daher werden adäquate Zellersatz-Strategien benötigt, die eine umfangreiche strukturelle und funktionelle Regeneration fördern. Neurale Stamm-/Vorläuferzellen (NPC), welche im adulten Nervensystem von Säugetieren einschließlich des Rückenmarks identifiziert wurden, stellen eine vielversprechende Quelle dar, um gerüst-bildende Astrozyten, remyelinisierende Oligodendrozyten sowie Neurone innerhalb des verletzten Rückenmarks zu ersetzen. Neurale Stamm-/Vorläuferzellen können entweder in oder um die Läsionsstelle herum durch die Gabe von geeigneten Molekülen stimuliert werden, mit dem Ziel verlorenes Rückenmarksgewebe intrinsisch (Stimulation des endogenen Zellersatzes) zu ersetzen. Alternativ können neurale Stammzellen aus kleinen Gehirn-/Rückenmarks-Biopsien isoliert, *in vitro* expandiert und schließlich in das verletzte Rückenmark transplantiert werden (Neurale Stammzell-Transplantation).

In einer kürzlich veröffentlichten Studie konnte gezeigt werden, dass mesenchymale Stammzellen (MSC) einen bisher noch nicht identifizierten Faktor sezernieren, welcher die oligodendrogliale Differenzierung von adulten hippocampalen Vorläuferzellen unter Ko-Kultur Bedingungen *in vitro* fördert und gleichzeitig die astrogliale Differenzierung der NPC inhibiert.

Basierend auf diesen Ergebnissen habe ich den Einfluss des Herkunftsortes der NPC in Bezug auf das Expressionsmuster spezifischer Differenzierungsmarker nach Inkubation mit konditioniertem Medium der MSC (MSC-CM) charakterisiert. Ich konnte zeigen, dass lösliche Faktoren der MSC die Expression von oligodendrozytären Markern in NPC unter *in vitro* Bedingungen induzieren, unabhängig von deren anatomischer Herkunft. Darüber hinaus führte die Inkubation von NPC mit konditioniertem Medium von Fibroblasten sogar zu einer noch höheren Expression des oligodendroglialen

Markers MBP und darüber hinaus zu einer signifikant niedrigeren Expression des astroglialen Markers GFAP. Diese Daten und die Tatsache, dass MSC und Fibroblasten beide mesenchymalen Ursprungs sind, deutet darauf hin, dass lösliche Faktoren sowohl der MSC als auch der Fibroblasten über den gleichen Signalweg wirken.

Im nächsten Schritt wurden NPC oder oligodendroglial vordifferenzierte NPC zusammen mit MSC auf hippocampale Schnittkulturen ausgesät. Hierbei zeigte sich, dass MSC auch in einer ZNS-organotypischen Umgebung die oligodendrogliale Differenzierung der transplantierten NPC fördern. Während das Überleben der ausgesäten NPC auf hippocampalen Schnitten gut war, war das Überleben der oligodendroglial-vordifferenzierten NPC sehr limitiert.

Anschließend habe ich NPC oder oligodendroglial vordifferenzierte NPC zusammen mit MSC in das intakte Rückenmark adulter Ratten transplantiert, um zu testen, ob die pro-oligodendrogene Aktivität der MSC auch *in vivo* aufrecht erhalten bleibt. Obwohl das Überleben der vordifferenzierten NPC sehr niedrig war, konnte eine signifikante Erhöhung der oligodendroglialen Differenzierung dieser Zellen im Vergleich zu NPC, die mit MSC ko-transplantiert wurden, festgestellt werden.

In weiterführenden Experimenten konnte durch die Ko-Transplantation von NPC mit MSC bzw. Fibroblasten ins läsionierte Rückenmark gezeigt werden, dass sowohl MSC- als auch Fibroblasten-enthaltende Transplantate, in der Lage waren den zystischen Läsionsdefekt, wie er charakteristischerweise nach einer Rückenmarksverletzung auftritt, zu ersetzen und ein unterstützendes zelluläres Gerüst zu bilden, um adulte NPC innerhalb der Läsion zu halten. Interessanterweise führte die Ko-Transplantation von NPC mit Fibroblasten zu einer erhöhten oligodendroglialen Differenzierung der ko-transplantierten NPC, wohingegen dieser Effekt bei einer MSC-NPC Ko-Transplantation nicht nachgewiesen werden konnte. *In vitro* Daten demonstrierten, dass BMP2 und BMP4 (Bone Morphogenic Protein 2 und 4), die nach einer Rückenmarksverletzung stark hoch reguliert werden, komplett dem MSC bedingten Effekt auf die oligodendrogliale Differenzierung der NPC, entgegenwirken. Demzufolge könnte die Neutralisation von BMPs

oder deren Signalkaskade, nötig sein, um die oligodendroglial Differenzierung durch MSC *in vivo* zu erhöhen.

Zusätzlich demonstriert diese Studie, dass die Transplantation von MSC ins läsionierte Rückenmark weder die Proliferation noch das Überleben von endogenen NPC beeinflusst. Allerdings führt die Transplantation von MSC schon innerhalb von drei Tagen nach einer Rückenmarksverletzung zu einer erhöhten Expression von oligodendroglialen Markern und nach einem Monat zu einer Verlagerung des Differenzierungsmusters der endogenen NPC in Richtung Oligodendroglia auf Kosten der astroglialen Differenzierung.

Zusammenfassend konnte in dieser Studie gezeigt werden, dass MSC eine pro-oligodendrogene Umgebung für NPC bereitstellen, welche auf hippocampale Schnitte bzw. ins intakte Rückenmark transplantiert wurden. Im Gegensatz dazu konnten diese Beobachtungen nach einer Rückenmarksläsion nicht mit ko-transplantierten NPC, sondern lediglich mit endogenen NPC rekapituliert werden.

Für jede zell-basierte Therapie, die in die Klinik transferiert werden soll, müssen geeignete bildgebende Verfahren etabliert werden, um morphologische Veränderungen darstellen zu können, die durch Zelltransplantationen ins verletzte Rückenmark hervorgerufen werden. Die Magnetresonanztomographie (MRT) stellt den Goldstandard dar, um das Rückenmarksparenchym nicht-invasiv zu visualisieren. Um die durch Zelltherapie induzierte morphologische Veränderungen zu validieren, habe ich Untersuchungen mittels eines klinischen Routine-3T MRT Scanner durchgeführt. In dieser Arbeit konnte gezeigt werden, dass es möglich ist, einen klinischen Routine-3T Kernspintomographen für die Bildgebung von Tieren zu nutzen, um nicht-invasiv pathologische Veränderungen, die nach einer Rückenmarksverletzung in der Ratte auftreten zu visualisieren und diese mit histologischen, strukturellen und Verhaltensveränderungen zu korrelieren.

1. Introduction

1.1. Neural stem cells and neurogenesis

1.1.1. History

In the last century the dogma existed that the adult central nervous system (CNS) is incapable of neurogenesis. In 1897 Schaper described the existence of “indifferent-cells” in the CNS. He suggested that these cells might exist lifelong and serve as material for regeneration processes after artificial or pathological substance deprivation in the CNS. But as far as he knew at this time, only a regeneration of neuroglia to recover the substance deprivation took place. It was Ramón y Cajal who first established the neuron doctrine (1913) by using the Golgi’s method. The neuron doctrine reveals that the nervous system consists of structural and functional units, the neurons. The neurons are individual cells, which have an axon and several dendrites and are not connected in a network. By using the newly invented ³H-Tymidine autoradiography method, Altman found in 1963 evidence of neurogenesis in the hippocampus (Altman 1963) and olfactory bulb (Altman 1965) of adult rats. Through this method dividing cells are labeled in the S-phase of the cell cycle by incorporating ³H-Tymidine into the DNA. But only the invention of the light microscopy as well as the usage of the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) provided structural evidence of neurogenesis in the adult mammalian brain (Nicholas B. Hastings 2000). But only when Kaplan and Hinds showed the existence of neurogenesis in the adult rat in 1977 by electron microscopy (Kaplan and Hinds 1977), the dogma of the “static brain” became outdated. They detected with ³H-Tymidine labeled neurons in the granular layers of dentate gyrus and olfactory bulb. And in 1984 Kaplan and Bell identified “mitotic neuroblasts with synapses” and axons on their cell bodies in the postnatal dentate gyrus (Kaplan and Bell 1984). But it was not until 1992, when Reynolds and Weiss published that “cells of the adult mouse striatum have the capacity to divide and differentiate into neurons and astrocytes, “ when the studies on adult stem cells and their potential to regenerate became a center of interest (Reynolds and Weiss 1992). A few

years later Weiss (Weiss et al. 1996) and Shihabuddin (Shihabuddin, Ray and Gage 1997) published that it is possible to isolate neural stem/progenitor (NPC) cells from the adult spinal cord, proliferate and expand them in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) and differentiate them into neurons, astrocytes and oligodendrocytes *in vivo*. Martens further found that NPC in the spinal cord lie next to the central canal (Martens, Seaberg and van der Kooy 2002). In 1998 Eriksson then published, the occurrence of “neurogenesis in the adult human hippocampus” and, that “the human brain retains the potential for self-renewal throughout life” (Eriksson et al. 1998).

1.1.2. Adult neural stem cells

Over the past decades, convincing evidence emerged that neurogenesis in the adult CNS is a continuous physiological process. Neurogenesis occurs in two regions: the subventricular zone (SVZ) of the lateral ventricles, where olfactory bulb neurons originate from, and the subgranular zone of the dentate gyrus of the hippocampus, which is a region important for learning and memory (Altman 1965, Kaplan and Hinds 1977, Kuhn, Dickinson-Anson and Gage 1996) (Fig.1.1).

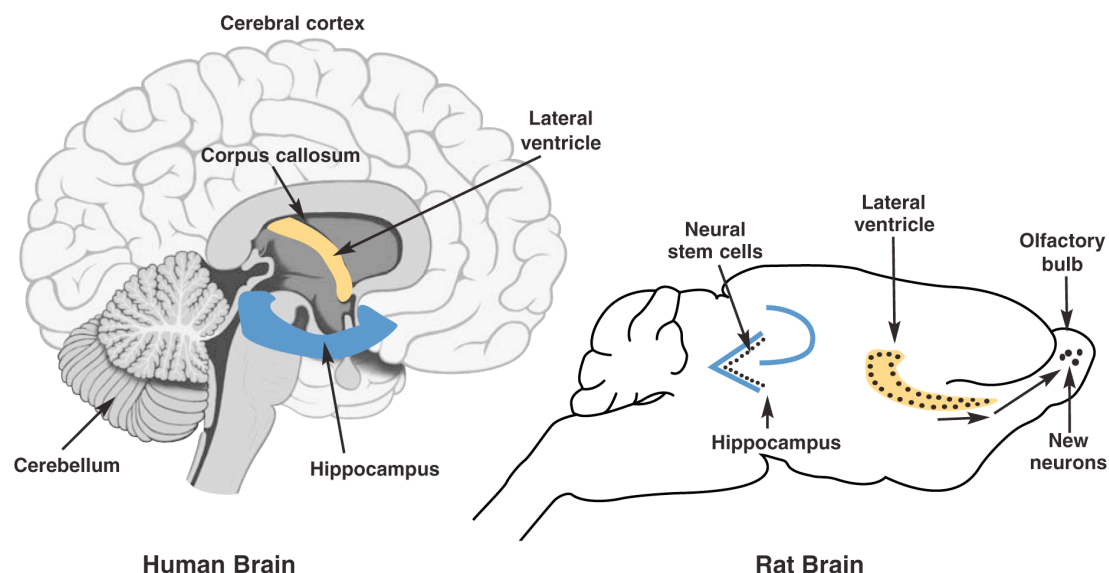


Figure 1.1. Sites of adult neurogenesis in the adult human and rat brain

Neurogenesis takes place in the subventricular zone (SVZ) of the lateral ventricles and in the dentate gyrus of the hippocampus in the adult brain. In the rat brain, neural progenitor cells from the SVZ migrate along the rostral migratory stream to the olfactory bulb, where they differentiate into neurons and integrate (Figure extracted from (Crews and Nixon 2003).

Additionally, recent studies indicated also the existence of NPC in other regions of the CNS, i.e. the striatum, neocortex and spinal cord (Palmer et al. 1999, Palmer, Ray and Gage 1995, Yamamoto et al. 2001, Weiss et al. 1996). New cells risen from the adult SVZ migrate along the rostral migratory stream to the olfactory bulb, where they differentiate into new interneurons (Kuhn and Svendsen 1999, Thomas, Gates and Steindler 1996, Lois and Alvarez-Buylla 1994, Doetsch and Alvarez-Buylla 1996). In the hippocampal neurogenesis, progenitor cells divide along the border of the hilus and the granule cell layer, whereas the daughter cells differentiate into granule and periglomerular neurons (van Praag et al. 2002, Kuhn and Svendsen 1999, Cameron et al. 1993, Cameron and McKay 2001, Lois and Alvarez-Buylla 1994). Adult NPC are multipotent *in vivo* as well as *in vitro* and characterized by their capacity to self-renew and divide indefinitely. They occur in the embryonic as well in the adult brain and have the potential to differentiate into the three major CNS lineages, neurons, astroglia and oligodendroglia (Gage 2000, Gotz and Huttner 2005, Kuhn et al. 1996) (Fig.1.2).

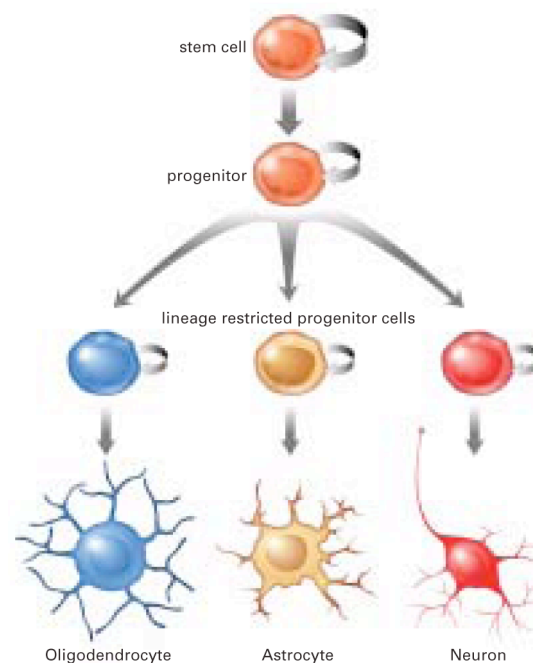


Figure 1.2. Potential of adult neural stem/progenitor cells

Adult NPC are multipotent and have the potential to self-renew. They can generate lineage restricted precursors cells, which further differentiate into the three major cell types of the CNS: neurons, astrocytes and oligodendrocytes. (Figure extracted from: www.helmholtz-muenchen.de/neu/Aktuelles/.../033_038_idg_akt.pdf).

Adult NPC cells are continuously generated and persist throughout the life span of mammals including humans (Kuhn et al. 1996, Eriksson et al. 1998, Kukekov et al. 1999). It is important to note that neurogenesis occurs either in a physiological mode or is exogenously modulated by external signals or pathophysiological processes. It is known, that external stimulants such as enriched environment and physical activity (van Praag, Kempermann and Gage 1999b, Kempermann, van Praag and Gage 2000, Gage 2000, van Praag et al. 1999a) or application of defined molecules such as bFGF (Kuhn et al. 1997), EGF (Palmer et al. 1995, Reynolds and Weiss 1992), vascular endothelial growth factor (VEGF) (Schanzer et al. 2004, Jin et al. 2002) or brain-derived neurotrophic factor (BDNF) (Chmielnicki et al. 2004, Gustafsson et al. 2003) and erythropoietin (Shingo et al. 2001) enhance neurogenesis, whereas stress acting through associated corticosteroid stress hormones (Tanapat et al. 2001, Gould et al. 1997, Cameron and Gould 1994, Duman, Malberg and Nakagawa 2001) or TGF-beta1 (Wachs et al. 2006) down-regulate neurogenesis. Although neurogenesis persists in the aged brain, its rate declines with age in rats (Kuhn et al. 1996), mice (Kempermann, Kuhn and Gage 1998), monkeys (Gould et al. 1999) and humans (Cameron and McKay 1999, Kukekov et al. 1999). Finally, CNS disease conditions such as seizures, stroke/hypoxia and traumatic brain injury represented by respective animal models induce neurogenesis (Kokaia and Lindvall 2003, Bengzon et al. 1997, Parent, Valentin and Lowenstein 2002, Jin et al. 2001, Ming and Song 2005, Parent 2003).

1.1.3. Culturing Methods - Adult neural stem cells in vitro

NPC of the of the adult CNS can be isolated from neurogenic regions like the subventricular zone (SVZ) or the hippocampus (Gage, Ray and Fisher 1995b, Reynolds and Weiss 1992) as well as from non-neurogenic regions like e.g. the spinal cord striatum and neocortex (Palmer et al. 1999, Palmer et al. 1995, Weiss et al. 1996). These cells can be isolated and propagated *in vitro* either as adherent monolayers on coated surfaces or as floating aggregates of NSC, the so-called neurospheres. Neurospheres are non-adherent, heterogeneous spherical structures, which are organized three-dimensional

fashion whereas they show a core of differentiated GFAP or β III-tubulin positive cells, which is surrounded by undifferentiated, proliferating cells (Campos 2004). By the usage of certain growth factors, it is further possible to augment and proliferate these cells in neurosphere cultures (Reynolds, Tetzlaff and Weiss 1992, Reynolds and Weiss 1992, Weiss et al. 1996). To maintain undifferentiated NSC *in vitro* it is necessary to culture them in the presence of EGF and/or bFGF (Roy et al. 2000, Wachs et al. 2003, Gritti et al. 1996). Recently, cell culture conditions were optimized/standardized to propagate neural stem cells at high expansion rates without losing multipotency and without promoting tumorigenicity (Wachs et al. 2003). The most common method to differentiate NSC is the withdrawal of the mitogens and/or adding different factors that induce some of the cells to develop into different lineages (e.g. serum) (Arsenijevic et al. 2001, Gage et al. 1995a, Johansson et al. 1999, Reynolds et al. 1992, Reynolds and Weiss 1992). The stem cells will efficiently differentiate in culture dishes into neurons, oligodendrocytes (the cells that insulate the electrical signals passing down axons in the nervous system) and astrocytes (another type of non-neuronal cell in the CNS) (Arsenijevic et al. 2001, Wachs et al. 2003, Kukekov et al. 1999, Roy et al. 2000). The isolation and proliferation of adult neural stem cells from the adult rodent CNS were replicated with adult CNS tissue derived from human subjects. Adult human neural stem cells were capable of generating astro-/oligodendroglial cells as well as neurons, thus confirming their multipotency (Arsenijevic et al. 2001, Johansson et al. 1999).

1.2. Spinal Cord Injury

Spinal cord injury in humans is in most instances caused by a blunt trauma that causes fracturation of the vertebral column with dislocation of bone fragments and consecutive contusion/compression of the spinal cord, which leads to the irreversible loss of function since the spinal cord has only limited capacity to self-renew. Motor control and sensory input is lost below the level of the lesion (Barnabe-Heider and Frisen 2008). Much of the prognosis and recovery depends on the location and severity of the injury. Some patients recover well and others may be paralyzed for life. Generally, patients

experience more paralysis when the injury is higher in the spinal column. Although spontaneous regeneration of lesioned fibres is absent in the adult CNS, many patients suffering from incomplete spinal cord injuries show significant functional recovery. This recovery process can go on for months, sometimes even years after the injury and probably depends on the reorganization of circuits (plasticity) that have been spared by the lesion (Raineteau and Schwab 2001).

1.2.1 Epidemiology of spinal cord injury

It is estimated that the annual incidence of spinal cord injury (SCI) in developed countries varies between 20 to 55 cases per million inhabitants, whereas motor vehicle accidents (42,1%), falls (26,7%), interpersonal violence (15,1%) and sports activities (7,6%) are main causes for SCI (Wyndaele and Wyndaele 2006, van den Berg et al. 2010) (Fig.1.3).

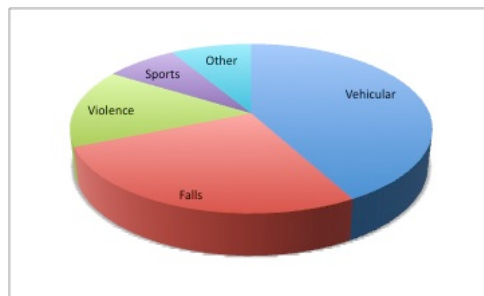


Figure 1.3. Etiology of spinal cord injury

(Figure adopted from: Spinal Cord Injury Statistics Updated June, 2009 (National Spinal Cord Injury Statistical Center (NSCISC), <http://www.fscip.org/facts.htm>).

Interestingly, the incidence rate in developing countries is much lower compared to that in developed countries (Chiu et al. 2010). Prevalence in acute SCI is defined as all persons with an SCI in a specified population at a particular point (Sekhon and Fehlings 2001). In the United States the prevalence of SCI is about 259,000 people. Since there are various studies where the investigators applied different methods concerning epidemiology of SCI, the evaluation of the existing studies is difficult. However in recent years the average age at injury did increase from around 30 to 40-50 years over the last years. In general males are more often affected by SCI than females, with a male-female ratio varying from 1.3:1 in Australia to up to 7.5:1 in Pakistan

(Chiu et al. 2010, van den Berg et al. 2010). Currently 80% of the SCI reported to the NSCISC the have occurred among males.

Spinal cord injury can be classified into 5 main categories based on the American Spinal Injury Association (ASIA) Impairment Scale (Fig.1.4). In a **complete injury** (ASIA A), the motor and sensory function is lost below the level of injury, whereas in an **incomplete injury** more or less sensation and/or movement below the level of the injury is retained (ASIA B through E). About 50 years ago, approximately two thirds of SCI were complete. Latest estimates show a shift towards incomplete injuries (55%). The majority of SCI affect the cervical segments, varying from 32 to 75% off the injuries resulting in tetraplegia and between 19 and 68% in paraplegia (van den Berg et al. 2010). Persons with tetraplegia have suffered injury to one of the eight cervical segments (C1-8) of the spinal cord with paralysis of both arms and legs. Those with paraplegia have lesions in the thoracic, lumbar, or sacral regions of the spinal cord, so that the function is lost only in the lower part of the body. Injuries can also be classified as traumatic or non-traumatic. **Traumatic injuries** are sudden causing immediate spinal cord damage and consecutive functional deficits. **Non-traumatic injuries** tend to develop more slowly from disorders compressing the spinal cord (cancer, hemorrhage, vertebral disk prolaps) and other diseases directly harming the spinal cord such as infections or ischemia. Nevertheless, even after a neurologically complete traumatic SCI, a variable rim of white matter closest to the surface of the spinal cord is usually spared (Kakulas 1999). Furthermore, functional recovery can be seen in many of the patients (Burns et al. 1997, Waters et al. 1995), as well as in animal models of incomplete SCI (Blight 1993, Little et al. 1999, Raineteau and Schwab 2001).

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- A (complete injury): No motor or sensory function is preserved in the sacral segments S4–S5.
- B (incomplete injury): Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4–S5.
- C (incomplete injury): Motor function is preserved below the neurological level, and more than a half of key muscles below the neurological level have a muscle grade of less than 3, which indicates active movement with full range of motion against gravity
- D (incomplete injury): Motor function is preserved below the neurological level, and at least a half of key muscles below the neurological level have a muscle grade of 3 or more.
- E (normal): Motor and sensory functions are normal.
-

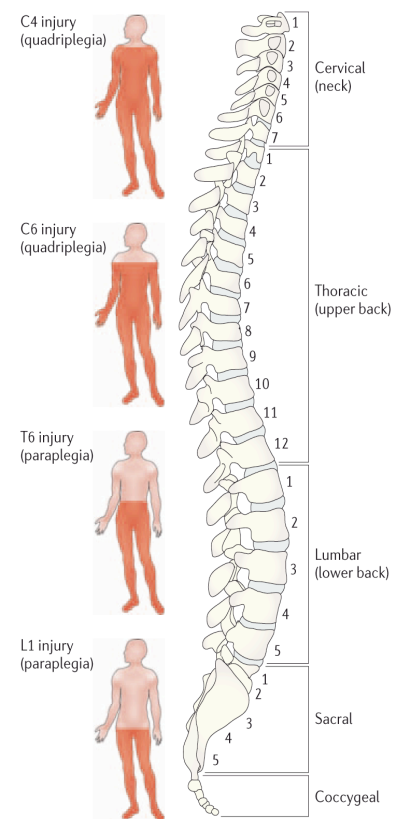


Figure 1.4. Classification of spinal cord injury severity using the American Spinal Injury Association (ASIA) Impairment Scale

SCI results in the disruption of motor control and sensory input below the level of the lesion (Figure extracted from (Thuret, Moon and Gage 2006)).

1.2.2. Pathomorphology of spinal cord injury

Spinal cord injury can be classified into four general types: 1) cord maceration, in which the morphology of the cord is severely distorted; 2) cord lacerations (gun shot or knife wounds), which results in a clear-cut disruption of the surface anatomy; 3) contusion injury, which leads to a central hematomyelia that may evolve to syringomyelia; and 4) solid cord injury, in which there is no central focus of necrosis as in contusion injury (Hulsebosch 2002, Bunge et al. 1993, Bunge, Puckett and Hiester 1997, Norenberg, Smith and Marcillo 2004).

There are three phases of the pathological response to SCI (Bunge et al. 1993, Bunge et al. 1997, Tator 1995, Tator 1996, Tator 1998). The **acute injury phase**, which starts at the moment of injury and may continue for hours or even days, depending on the severity of the injury. The initial force of the traumatic insult, evoked by compression, laceration, contusion, or stretch

injury of the cord (Anthes, Theriault and Tator 1996, Anthes, Theriault and Tator 1995), causes an immediate mechanical damage, affecting the neurons, axons and blood vessels of the spinal cord. This acute injury triggers a number of pathophysiological events. A shift of electrolytes in the cells at the lesion center is occurring, increasing the intracellular Na^+ -concentration. The increase of K^+ into the extracellular space results from an injury-induced block of axonal conduction (Eidelberg, Sullivan and Brigham 1975), which contributes to the state known as spinal shock. Spinal shock usually recovers within 24 hours. It represents a generalized failure of circuitry in the spinal neural network characterized by the temporary loss of spinal activity (Hulsebosch 2002).

Another common pathological feature of acute SCI is an early hemorrhage with localized edema, loss of microcirculation, axonal and neuronal necrosis and demyelination (Hulsebosch 2002, Tator and Fehlings 1991).

In the **secondary injury or recovery phase**, refers to a period during which function returns, starting within hours and extending for months or even years after the initial injury. The pathophysiological alterations (ischemic cellular death, electrolytic shifts, and edema) initiated in the acute injury phase persist leading to a progressive loss of axons and neurons. Additionally, apoptosis occurs, involving reactive gliosis. As a result, the size of the initial lesion grows to a notably larger cystic lesion defect (Hulsebosch 2002, Collins 1983).

Finally, the **chronic injury phase** occurs over a period of days to years and refers to a time when functional recovery has reached a plateau and the spinal cord anatomy has been irreversibly changed (Collins 1983). Necrosis as well as the continuing apoptosis at the lesion center has leads to massive tissue degeneration and the development of a cystic lesion cavity (Kao and Chang 1977). These effects induce the activation of microglia and the invasion of macrophages and fibroblasts, causing in the replacement of white and grey matter by the glial scar tissue. Demyelination is caused by apoptosis of oligodendrocytes and results in rearrangements of ion channels, which further leads to conduction deficits of axons that have survived the initial trauma, (Hulsebosch 2002, Shuman, Bresnahan and Beattie 1997, Barnabe-Heider and Frisen 2008). In some cases, the initial lesion cyst is enlarged due

to the flow cerebrospinal fluid (CSF) into the resulting cavity, a condition called syringomyelia (Potter and Saifuddin 2003). In many cell types, permanent hyperexcitability develops, which results in chronic pain syndromes (Christensen et al. 1996, Christensen and Hulsebosch 1997). It is now generally accepted that the mechanisms which occur after an acute spinal cord injury is a two-step process involving the primary and secondary injury mechanism, even though it is difficult to separate them temporarily (Tator and Fehlings 1991).

1.2.2.1. Primary injury mechanisms

The mechanisms of the primary injury are caused by the acute compression or laceration of the spinal cord, due to the initial mechanical force of the traumatic insult. This mechanical impact can involve the displacement of bony fragments or disc material into the spinal cord during burst fracture or fracture dislocation of the spine (Tator 1995, Tator and Fehlings 1991). A less frequent occurring injury type is the direct transection of the spinal cord which often occur after knife wounds or gun shots (Sekhon and Fehlings 2001). Several injury mechanisms occur, including the damage cell membranes, mechanical damage of the spinal cord axon pathways and damage to the vasculature of the cord.

1.2.2.2. Secondary injury mechanisms

The secondary injury is initiated by damage to the cell membrane, resulting in ionic derangement, which leads to the progressive destruction of the spinal cord (Borgens 2003). Progressive loss of axons and neurons take place in massive additional damaging processes after the impact to the cord, initiated by the primary injury (Collins 1983, Sandler and Tator 1976a, Sandler and Tator 1976b). These secondary injury mechanisms are autocatalytic and can occur over many days, even weeks. They involve numerous biological processes, cellular responses and vascular events that develop over minutes to hours after the primary injury and lead to demyelination and scar formation (Borgens 2003).

1.2.2.2.1. Vascular events

Acute SCI causes within minutes after the primary injury numerous vascular changes. The normal autoregulation in the spinal cord is lost due to systemic hypotension, a state, which is called neurogenic shock (Senter and Venes 1979, Tator and Fehlings 1991, Young, DeCrescito and Tomasula 1982). The impairment of the spinal autoregulation additionally leads to a reduction of blood flow in the spinal cord, resulting not only in vasospasm and thrombosis but also in the reduction of microcirculation and lack of perfusion (Tator 1991, Senter and Venes 1979). These vascular disruptions are associated with post traumatic ischemia especially in areas adjacent to hemorrhage and lead to infarction and necrosis, which is more severe in grey than in white matter tissue (Sandler and Tator 1976a, Tator 1991, Tator and Fehlings 1991, Nelson et al. 1977). Moreover, ultrastructural alterations of the blood-brain barrier consist of

endothelial changes leading to edema formation and glial swelling (Goodman, Bingham and Hunt 1976).

1.2.2.2.2. Biochemical changes

The release of excitatory amino acids contributes to secondary tissue damage after traumatic spinal cord injury. It has been described that the excitatory amino acid neurotransmitter glutamate has an excitotoxic effect following SCI (Demediuk, Daly and Faden 1989, Westerberg et al. 1987). The increased extracellular glutamate concentration (Benveniste et al. 1984) leads to the subsequent activation of the glutamate receptor N-methyl-D-aspartate (NMDA) in the neuronal cell membrane, which has been shown to open the receptor dependent NMDA channels resulting in the massive influx Ca^{2+} into the cell (Choi 1988, Tator 1995, Tator and Fehlings 1991). In addition, increase of intracellular Na^+ initiates the release of Ca^{2+} from intracellular stores such as the endoplasmic reticulum or mitochondria, through the reversed action of $\text{Na}^+/\text{Ca}^{2+}$ exchanger by pumping out Ca^{2+} from intracellular stores into the cytoplasm in reverse of Na^+ (Stys, Waxman and Ransom 1991a, Stys, Waxman and Ransom 1991b, Borgens 2003). Increased intracellular Ca^{2+} in turn activates calcium-dependent proteases, leading to

the degradation of myelin proteins and cytoskeletal components including neurofilaments (Banik et al. 1982, Banik et al. 1984). The cell membrane is destabilized, which causes impaired axoplasmatic transport (Schlaepfer and Bunge 1973). Furthermore, the mitochondrial function is also impaired, by what Na^+ streams into the mitochondria, which in turn reduces the ATP production, resulting not only in a reduced Na^+/K^+ -ATPase activity, but also in the release of cytochrome C, which induces apoptotic cell death by caspase 3 activation (Narita et al. 1998). In addition, elevated Ca^{2+} levels activate the calcium-dependent enzymes phospholipase C and kinase C, which induce cell lysis and necrosis (Tator 1995, Tator and Fehlings 1991, Muller et al. 1999, Hulsebosch 2002).

The collapse of oxygen metabolism within the cell, coupled with specific enzymatic catalysis, leads to the production of highly reactive oxygen metabolites such as superoxide anions, hydrogen peroxide and hydroxyl radicals (Dyken 1994). These so called free radicals are cytotoxic and promote lipid peroxidation, which contributes to the breakdown of ionic membrane gradients as well as to the disruption of the cell membranes (Yamamoto et al. 1983).

1.2.2.2.3. Cellular events

Following CNS injury, a pronounced cellular inflammatory reaction occurs which is characterized by the activation of resident and recruited immune cells. In regions rostral or caudal to the epicenter, prolonged activation of inflammatory cells occurs preferentially in white matter and primarily consists of activated microglia and astrocytes (Dusart and Schwab 1994, Popovich, Wei and Stokes 1997).

Within 6 hours after the primary injury, a significant neutrophil infiltration in the lesion site appears and after 24 h they reach their highest cell density. The following days are characterized by a gradual replacement of neutrophils by macrophages. Proliferation and recruitment of macrophages and microglial cells become predominant 2 days after injury. At day 4 to 7, macrophage-like cells remove tissue debris by phagocytosis. This process, together with a reaction of the surrounding astrocytes, finally leads to the formation of

cavities, which are surrounded by the glial scar. Besides the restoration of tissue homeostasis, neutrophils are able to release oxygen radicals as well as a variety of enzymes and contribute therefore to secondary extension of the lesion (Dusart and Schwab 1994). Already one hour after the lesion, microglial cells and astrocytes become activated. Some activated microglia convert to a macrophage phenotype that is referred as „brain-macrophage“ and remove axon and myelin debris by phagocytosis (Popovich et al. 1997). Furthermore, activated microglia function as antigen presenting cells to mediate the T-cell response (Schmitt et al. 2000). It has been shown, that macrophages and lymphocytes infiltrate the injured spinal cord tissue several days after the initial injury (Popovich et al. 1997). This infiltration together with the persisting immune cell activation has both beneficial and destructive effects on the functional outcome after SCI. The removal of axon and myelin debris has positive effects, since it is known that adult CNS myelin contains axon growth inhibitors (Hauben et al. 2000). On the other hand, SCI induces the expression of pro-inflammatory cytokines like the tumor necrosis factor-alpha (TNF-alpha) (Bethea et al. 1999) as well as the production of nitric oxide (NO) by neuronal nitric oxide synthase which in turn are involved in the secondary damage to neuronal tissue (Wada et al. 1998).

Reactive astrocytes are also present in the acute lesion but more prominent at 7-28 days after the injury. These cells are observed only in the surrounding outer ring of spared white matter at the lesion site and in tissue adjacent to the lesion but are mainly absent in the lesion site (Popovich et al. 1997, Eng, Reier and Houle 1987). Reactive astrocytes can be identified by their increased immunoreactivity for the intermediate filament protein GFAP and vimentin (Eng et al. 1987, Miller et al. 1986). An important property of reactive astrocytes is that they surround the lesion cavity by forming a wall of scar tissue in response to injury in the CNS - a process called reactive gliosis (Miller et al. 1986). Unlike fibroblasts, which form scars in nonneural tissues by secreting large amounts of collagenous extracellular matrix, astrocytes form scars by producing numerous extracellular matrix proteins, including the axonal growth inhibiting chondroitin sulfate proteoglycan (CSPG) proteins like versican, phosphocan, NG2, and neurocan (McKerracher et al. 1994, Bignami et al. 1972, Miller et al. 1986, Hulsebosch 2002). But meningeal fibroblasts can

also invade the lesion, forming basement membranes, probably in collaboration with astrocytes (Bernstein et al. 1985, Feringa, Vahlsing and Woodward 1984, Schwab and Bartholdi 1996). It is known that astrocytes are a heterogeneous class of cells (Miller and Raff 1984, Raff et al. 1983), whereby the type-1 astrocytes, together with activated microglia/macrophages, are responsible to form the glial scar tissue in the white matter surrounding the lesion site (Miller et al. 1986, Dusart and Schwab 1994). The presence of growth-inhibitory proteins like CSPGs leads to the formation of the glial scar, which represents a barrier to axonal growth (Liuzzi and Lasek 1987, Rudge and Silver 1990, Hulsebosch 2002). Nevertheless, reactive astrocytes also play an important role in the repair of the blood-brain barrier and are essential for wound healing (Faulkner et al. 2004). Moreover, reactive astrocytes exhibit important protective functions for neurons and oligodendrocytes by producing numerous growth factors and cytokines. They also preserve motor functions and play fundamental roles in regulation leukocyte infiltration after mild or moderate SCI (Faulkner et al. 2004, Bush et al. 1999). Therefore, reactive astrocytes play a bivalent role after injuries to the CNS. On the one hand, they stabilize the injured tissue during the secondary injury phase, but inhibit axonal regeneration on the other hand.

After an injury to the spinal cord, axonal loss takes place in the ascending fiber tracts above the lesion and in descending fiber tracts below the lesion. The distal segment of a nerve fiber (the part no longer connected to the neuronal cell body) retracts from postsynaptic neurons and undergoes secondary or Wallerian degeneration, and although the proximal segment typically survives, it is unable to successfully regenerate (Bradbury and McMahon 2006, Franklin and French-Constant 2008). Wallerian degeneration happens in both the central and the peripheral (PNS) nervous system and is characterized by segmentation of the myelin and results in atrophy and destruction of the axon (Basiri and Doucette 2010). Although there are some similarities in the molecular and cellular pattern of Wallerian degeneration in the PNS and CNS, like the degeneration of myelin sheaths, (Franson and Ronnevi 1984, George and Griffin 1994), the removal of axonal and myelin debris by microglia and astrocytes is greatly delayed in the CNS and occurs over a much longer time frame than seen in a peripheral nerve

(Griffin et al. 1992, Basiri and Doucette 2010, George and Griffin 1994, Vargas and Barres 2007). The poor efficiency in removing axonal and myelin debris arising as a result of Wallerian degeneration in the CNS vs. the PNS is likely due to differences in the microglial and macrophage responses (George and Griffin 1994, Vargas and Barres 2007). Following transection of axons, the continuing axoplasmic flow leads to the formation of terminal clubs in both proximal and distal stumps of the lesioned axons (Kao, Chang and Bloodworth 1977a, Kao, Chang and Bloodworth 1977b). The dissolution of the terminal clubs results in autolysis of spinal cord tissue and the subsequent cavitation of the cord due to the release of hydrolytic lysosomal enzymes (Kao et al. 1977a, Kao et al. 1977b).

The characterized cellular and vascular events as well the mentioned biological processes are part of the secondary injury phase and also affect cells of the spinal cord, which at first survived the initial injury. Secondary cell death occurs, whereas most apoptotic cells are present in the area of the lesion epicenter (Yong et al. 1998, Tator 1991). Apoptosis of oligodendrocytes adjacent to spared axons causes chronic demyelination, which often represents the morphological correlate for the delayed loss of function after spinal cord injury (Blight 1994, Totoiu and Keirstead 2005, Blight 1985).

1.2.3.1. Extrinsic Inhibitors and Barriers of Regeneration

1.2.3.1.1. Cavity formation

After the initial injury and following necrosis as well as apoptosis, a fluid filled cavity develops, resulting in the loss of grey and white matter (Kao and Chang 1977). The initial cyst can be enlarged and expanded to additional spinal cord segments causing additional cell death and increased loss of function (Potter and Saifuddin 2003, Greitz 2006). The formation of the cystic cavity displays a physical barrier to spontaneous regeneration (Willerth and Sakiyama-Elbert 2008). Cell therapies have shown beneficial effects, by providing trophic support for cell migration or by secreting factors that promote axon regeneration into the cavity. The infusion of e.g. mesenchymal stromal cells (MSC) into the cerebrospinal fluid (CSF) of the spinal cord resulted in a smaller lesion cavity and the improvement of behavioral function compared to controls (Ohta et al. 2004, Wu et al. 2003).

Table 1.1. Secondary injury mechanisms involved in the Pathophysiology following acute spinal cord injury

Vascular events

Mechanical disruption of capillaries and venules
 Disruption of blood brain barrier
 Loss of autoregulation
 Hemorrhage (especially in grey matter)
 Loss of microcirculation / Reduction of spinal cord blood flow (thrombosis, vasospasm)
 Post traumatic ischemia
 Edema

Biochemical changes

Uncontrolled excitatory amino acid release → Excitotoxicity (glutamate)
 Ca^{2+} influx into cells
 Na^{+} influx into mitochondria
 Collapse of oxidative metabolism and decreased ATP production
 Cytochrome C release
 Free radical overproduction
 Lipid peroxidation

Cellular events

Invasion of immune cells (neutrophils)
 Microglia activation
 Reactive Astrogliosis
 Wallerian degeneration
 Rupture of terminal clubs resulting in hydrolytic enzyme release
 Apoptosis of glial cells
 Invasion of fibroblasts

1.2.3.1.2. Glial scar formation

One of the most potent obstacles for axonal recovery after SCI represents the glial scar formation. Many different types of cells such as microglia, macrophages, oligodendrocyte precursors, and meningeal cells are recruited to the injury site at different time points, but the glial scar consists predominately of reactive astrocytes with additional contributions of extracellular matrix (ECM) molecules, especially CSPGs and during the scar formation, inflammatory cells (Fawcett and Asher 1999, Schwab and Bartholdi 1996, Gallo, Bertolotto and Levi 1987, Katoh-Semba et al. 1995). The scar is comprised of two main zones, the lesion core, where meningeal fibroblasts, vascular endothelial cells, and frequently oligodendrocyte precursors (OPC) can be found, and the surrounding area consisting of reactive astrocytes, OPC, and microglia. Additionally, debris from the damaged tissue, axons, and degenerating myelin is present in the scar. The lesion core represents usually an utterly barrier for axon regeneration (Fawcett 2006, Reier and Houle 1988). When re-growing axons enter the environment of the glial scar they form so

called dystrophic endbulbs (Silver and Miller 2004, Liuzzi and Lasek 1987, Rudge and Silver 1990). Furthermore, the astrocytes are tightly packed and attached to one another by gap and tight junctions surrounded by ECM, so that they separate the injured tissue from its surrounding (Fawcett and Asher 1999, Reier and Houle 1988, Eng et al. 1987). After an injury, many astrocytes become hypertrophic (Dusart and Schwab 1994, Bunge et al. 1994) and start to up-regulate glial fibrillary protein (GFAP), vimentin and nestin as well as inhibitory molecules (Fawcett and Asher 1999, Silver and Miller 2004). The most important inhibitory molecules produced by reactive astrocytes are CSPGs (McKeon, Juryneec and Buck 1999, Willerth and Sakiyama-Elbert 2008, Rolls, Shechter and Schwartz 2009). They form a relatively large family, which includes aggrecan, brevican, neurocan, NG2, phosphacan and versican, all of which have chondroitin sulphate side chains. CSPGs furthermore consist of a core protein and one or more covalently attached glycosaminoglycan chains (GAGs) and are secreted by almost all cell types at the injury site (especially astrocytes) (Katoh-Semba et al. 1995, Tang, Davies and Davies 2003, Asher et al. 2000, Asher et al. 2002, Davies et al. 2004). The sulphated GAGs play an important role in the inhibitory effects on axonal growth of CSPGs, because it has been demonstrated in many studies that the removal of the GAG chains, e.g. by treatment with the enzyme chondroitinase ABC (ChABC), promotes axon growth (McKeon, Hoke and Silver 1995, Smith-Thomas et al. 1995, Smith-Thomas et al. 1994, Curinga et al. 2007, Silver and Miller 2004).

Besides proteoglycans, several other axon growth inhibitors have been identified in the glial scar tissue. The secreted protein semaphorin 3 (Sema 3), is expressed in invading fibroblasts and acts as a chemorepellent through its high-affinity receptor neuropilin (De Winter et al. 2002, Pasterkamp et al. 1999, Silver and Miller 2004). In addition, Sema 5A, can bind both to CSPG GAG chains, resulting in changes of the semaphorin from an axon growth permissive configuration to a growth inhibitory one (Kantor et al. 2004, Fawcett 2006). It also has been demonstrated that members of the eph/ephrin family of tyrosine kinases and their ligands are up-regulated after injury. The ephrin-B2 expression is increased in astrocytes whereas the expression of the receptor EPHB2 is increased in meningeal fibroblasts, which invade the

injured spinal cord. The binding of ephrin-B2 with its receptor leads to the formation of the so called glial/mesenchymal scar and inhibits axon regeneration (Bundesen et al. 2003, Silver and Miller 2004, Fawcett 2006, Feringa et al. 1984). Moreover, the secreted Slit proteins together with their high-affinity receptor glypican-1, a heparan sulfate proteoglycan, are up-regulated in reactive astrocytes and show repulsive effects for axon elongation (Hagino et al. 2003, Silver and Miller 2004).

After the disruption of the blood-brain barrier (BBB) as a result of SCI, the formation of the glial scar occurs, which is induced by the invasion of activated microglia and the influx of normally absent molecules in the damaged spinal cord tissue (Preston, Webster and Small 2001). Potential triggers for the induction of reactive gliosis are the transforming growth factor- β (TGF β) and interleukin-1, which are secreted by astrocytes and macrophages. They have shown to play an important role in the transformation of normal astrocytes into reactive astrocytes (Moon and Fawcett 2001, Silver and Miller 2004, Rolls et al. 2009). Furthermore, the interaction between the inflammatory cytokine interferon- γ and the basic fibroblast growth factor 2 (FGF2) take place in the induction of the glial scar (Yong et al. 1991, Silver and Miller 2004).

In addition to preventing recovery, the glial scar also provides several beneficial functions. It takes place in stabilizing the spinal cord parenchyma after injury, by creating a scaffold (Silver and Miller 2004, Rolls et al. 2009). Some populations of astrocytes even support axon re-growth and protect neurons directly from nitric oxide toxicity (Yiu and He 2006, Faulkner et al. 2004, Rolls et al. 2009, Chen et al. 2001). By producing and secreting CSPGs in the lesion area, activated astrocytes isolated the injury site by building a barrier for potentially harmful molecules in order to minimize the area of cellular degradation and balance the inflammatory response (Chen et al. 2001, Yiu and He 2006, Rolls et al. 2009, Roitbak and Sykova 1999). Furthermore, astrocytes provide trophic support at the injury site (do Carmo Cunha et al. 2007, White, Yin and Jakeman 2008). Therefore, the balance between inhibitory and beneficial effects of the glial scar fundamentally influences the ability of axons to regenerate.

1.2.3.1.3. Myelin based inhibitors

Several axon growth inhibitors, which are released by damaged oligodendrocytes, including Nogo, Myelin-Associated Glycoprotein (MAG), Oligodendrocyte Myelin glycoprotein (OMgp) and tenascin-R, have been identified in the CNS myelin (Willerth and Sakiyama-Elbert 2008, Busch and Silver 2007, McGee and Strittmatter 2003, Kottis et al. 2002, Salzer, Holmes and Colman 1987, McKerracher et al. 1994). Nogo exists in three isoforms, Nogo-A, Nogo-B and Nogo-C, but the predominant isoform expressed in the CNS is Nogo-A, which consists of two inhibitory domains, that are primarily associated with the endoplasmatic reticulum of oligodendrocytes (GrandPre et al. 2000). The extracellular domain, also referred to as Nogo-66, contains 66 amino acids and is located at the C-terminus and the Amino-Nogo-A domain is located at the N-terminus (GrandPre et al. 2000, Willerth and Sakiyama-Elbert 2008, Wang et al. 2002, Schwab, Tuli and Failli 2006b, Oertle et al. 2003).

The myelin-associated transmembrane glycoprotein MAG was the first characterized inhibitor from myelin *in vitro* (McKerracher et al. 1994, Mukhopadhyay et al. 1994). MAG is a member of the sialic acid-binding subgroup of the Ig superfamily and selectively localized in periaxonal Schwann cell and oligodendroglial membranes of myelin sheaths, suggesting that it functions in glia–axon interactions in both the PNS and CNS (Quarles 2007, McKerracher et al. 1994, Salzer et al. 1987). *In vivo*, however, loss of MAG function fails to promote extensive regeneration (Bartsch et al. 1995, Li et al. 1996).

The OMgp is a glycosylphosphatidylinositol-anchored protein expressed by neurons and oligodendrocytes in the CNS. It is a member of leucine-rich repeats protein family and involved in growth cone collapse and inhibition of neurite outgrowth (Wang et al. 2002, Vourc'h and Andres 2004).

The three proteins (Nogo-A, OMgp, and MAG) all bind to the Nogo receptor NgR, a glycosylphosphatidyl-anchored (GPI-anchored) protein (Fournier, GrandPre and Strittmatter 2001). A receptor complex, comprised of the NgR, the 75 receptor and LINGO-1, transduces the signals from all these inhibitors *in vitro*. Downstream of these inhibitors, the activation of small GTPase RhoA

and its effector Rho-kinase has been shown to mediate the inhibition of neurite outgrowth (Willerth and Sakiyama-Elbert 2008, Niederost et al. 2002, Liu et al. 2002, Fournier et al. 2001, Oertle et al. 2003).

The role of these proteins as potent inhibitors of neurite outgrowth and growth cone collapse has been identified in various culture experiments (McKerracher et al. 1994, Chen et al. 2000, GrandPre et al. 2000, Prinjha et al. 2000), but the exact role *in vivo* remains unclear. These proteins can be neutralized to improve functional outcome after SCI through the application of Nogo specific antibodies such as the monoclonal antibody IN-1 (Thallmair et al. 1998, Z'Graggen et al. 1998, Chen et al. 2000, Bregman et al. 1995, Caroni and Schwab 1988), agonists such as Nogo-66 (McGee and Strittmatter 2003), using enzymatic removal of the receptor (Liu et al. 2002) or by knocking down expression of the Nogo receptor that binds these proteins (Willerth and Sakiyama-Elbert 2008, Li et al. 2005). The inhibition of NgR signaling after SCI through the delivery of antibodies against Nogo leads to long-distance re-growth of only a small proportion of corticospinal axons. Nevertheless, axon regeneration and anatomical plasticity can be stimulated in corticospinal pathways in order to enhance functional recovery in animal models of SCI (Brosamle et al. 2000, Bregman et al. 1995, Li and Strittmatter 2003, Thallmair et al. 1998, Schnell and Schwab 1990). Even though the axons of Nogo-A/B deficient mice showed enhanced neurite outgrowth *in vitro*, the knockout of either specific or all three Nogo variants did not or at best result in a very limited enhanced regeneration of corticospinal tract (CST) axons following a dorsal column lesion (Lee et al. 2009, Zheng et al. 2003, Kim et al. 2003, Simonen et al. 2003), suggesting that Nogo alone is not sufficient to induce extensive axon regeneration.

Tenascin-R is an extracellular matrix glycoprotein present in the CNS, particularly in white matter. It is produced by oligodendrocytes, and up-regulated after injury to the CNS (Milev et al. 1998). It has been demonstrated that tenascin-R can inhibit axon growth and restrict motor neuron innervations (Apostolova, Irintchev and Schachner 2006, Becker et al. 1999, Willerth and Sakiyama-Elbert 2008, Fawcett and Asher 1999). In addition to its direct effects on axon growth, tenascin has binding sites for most of the inhibitory CSPGs (Rauch et al. 1997, Fawcett and Asher 1999). Tenascin-R deficient

mice showed improved functional recovery after SCI compared to wild type mice, suggesting that knocking down its expression could be used as a therapeutic intervention (Willerth and Sakiyama-Elbert 2008, Apostolova et al. 2006).

1.2.3.2. Intrinsic factors limiting regeneration

1.2.3.2.1. Genes associated with regeneration

The different intrinsic abilities of lesioned CNS neurons contribute significantly to the poor regenerative capacity of the CNS. Various molecular and biochemical changes take place in neuronal cells in response to axotomy. The up-regulation of neurite growth and regeneration associated genes (RAGs) such as GAP-43 (growth associated protein-43) (Bomze et al. 2001, Ramer, Priestley and McMahon 2000, Ramer et al. 2002), CAP-23 and SPRR1A (small proline-rich repeat protein 1A) (Bonilla, Tanabe and Strittmatter 2002) as well as the up-regulation of cell surface adhesion molecules, in particular those of the L1 family and NCAM, seems to be important for an effective axonal regeneration (Maier and Schwab 2006, Kwon et al. 2007, Bulsara et al. 2002). In contrast to PNS injuries, SCI and other CNS lesions activate RAGs in only in some injured neurons, which may be one reason for the failure of most axons of the CNS to regenerate (Broude et al. 1997, Jung, Petrusch and Stuermer 1997, Bulsara et al. 2002, Schaden, Stuermer and Bahr 1994, Schreyer and Skene 1993). It has been demonstrated that overexpression of RAGs can induce axonal regeneration (Bomze et al. 2001, Ramer et al. 2002, Ramer et al. 2000). This indicates that a successful regeneration of axotomized CNS neurons is correlated with the expression levels of RAGs (Bulsara et al. 2002, Becker et al. 1998, Kwon et al. 2007).

1.2.3.2.2. Trophic support

Neurotrophic factors such as nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) have been shown to play key roles in neuronal survival and differentiation during the development of the CNS and PNS. In addition, they are involved in the maintenance of a neuronal phenotype and influence synaptic functions (Lindsay et al. 1994, Kobayashi et

al. 1997, Schnell et al. 1994, Davies 1994, Lewin and Barde 1996). In contrast to PNS neurons, CNS neurons lack sufficient expression of neurotrophins, which can result in axotomy-induced atrophy of rubrospinal and corticospinal neurons (Kobayashi et al. 1997, Giehl and Tetzlaff 1996). On the other hand, the application of neurotrophins at the cell body of the red nucleus prevented atrophy of rubrospinal neurons (Bretzner et al. 2008, Kobayashi et al. 1997). Moreover, it has been demonstrated in several studies that local administration of neurotrophins can enhance sprouting of CNS axons (Oudega and Hagg 1999, Schnell et al. 1994, Tuszynski et al. 1994, Xu et al. 1995a, Ye and Houle 1997). Furthermore, the administration of BDNF and NT-4/5 has shown to augment the poor intrinsic regenerative capabilities of CNS neurons by reversing neuronal atrophy and stimulating the expression of GAP-43 in order to promote functional and structural recovery. Taylor et al. showed that the delivery of an NT-3 gradient rostral to the injured spinal cord promoted short-distance axonal regeneration (Taylor et al. 2006), whereas the application of a BDNF gradient induced long, directional axonal growth (Bonner et al. 2010). These studies imply that the administration of neurotrophic factors represents a potential strategy to induce axonal recovery.

1.2.3.3. Demyelination

Demyelination due to the loss of oligodendrocytes and the resulting conduction deficits in surviving axons, also contributes significantly to functional deficits after SCI (Gledhill, Harrison and McDonald 1973, Hulsebosch 2002). Since the CNS exhibits only a very limited capacity for remyelination, and the terminal differentiation from endogenous NPC into mature oligodendrocytes is often not sufficient, cell replacement strategies are a promising strategy in order to promote remyelination.

Possible candidates are cells forming PNS type myelin such as Schwann cells and olfactory ensheathing cells (OEC), which have been shown to form functionally relevant myelin with improvement of saltatory nerve conduction after transplantation into various CNS sites (Weidner et al. 1999a, Blakemore and Crang 1985, Honmou et al. 1996, Sasaki et al. 2004, Akiyama et al. 2004). OPC/oligodendrocytes responsible for myelination in the CNS, also

promoted remyelination and functional improvement after transplantation (Windrem et al. 2004, Archer et al. 1997, Brustle et al. 1999). Another approach to improve functional outcome after SCI would be to protect the remaining endogenous oligodendrocytes or to stimulate endogenous NPC to differentiate into mature oligodendrocytes.

1.2.4. Strategies to induce recovery after spinal cord injury

The ultimate goal would be to achieve both, functional and structural recovery after injuries to the spinal cord. In order to measure functional recovery, behavioral as well as electrophysiological test need to be performed. To determine structural recovery, like tissue repair, axonal growth, remyelination or synapse formation, descriptive tests to identify the integrity of the injured tissue can be performed. The application of several strategies to improve the outcome after SCI, are described in the following sections.

1.2.4.1. Neuroprotection – Reduction of secondary damage

Traumatic injury to the spinal cord leads to the loss of grey matter and white matter (WM) tissue. The functional deficits produced by SCI are mainly caused by the loss of white matter, particularly by disrupting the long tracts of descending and ascending axon pathways (Blight and Decrescito 1986, Noble and Wrathall 1989).

Interestingly, the sparing of WM is highly correlated to the recovery of hindlimb locomotion after SCI (Blight 1991, Basso, Beattie and Bresnahan 1996). The pathophysiological changes emerging during the secondary injury phase are involved in the increased white matter loss over time after the initial injury (Tator 1995, Rosenberg and Wrathall 1997). Therefore, it would be important to apply therapeutic interventions before the secondary injury phase starts.

Improved functional outcome after SCI may be elicited by neuroprotective approaches that limit secondary tissue loss and thus the loss of function. Removing or blocking inhibitory molecules such as Nogo-A, MAG, OMgp, CSPGs, collagen, semaphorins or ephrins, in the spinal cord lesion area has been shown to induce tissue sparing and functional recovery after SCI (Liu,

Chen and Tao 2008, Fawcett 2006, Schwab et al. 2006a). Moreover, the reduction of the release of glutamate, cytochrome C, Ca^{2+} , Na^{+} , or free radicals also have beneficial effects on functional and structural outcome (Schwab et al. 2006a, Teng et al. 2004, Teng and Wrathall 1997). Apart from cyclooxygenase inhibitors (Hurley, Olschowka and O'Banion 2002, Schwab et al. 2004), which have an anti-inflammatory effect and reduce the formation of free radicals, recently erythropoietin (EPO) has been discovered as a glia- and neuroprotective drug (Goldman and Nedergaard 2002, Gorio et al. 2002). Beside its anti-inflammatory role (Agnello et al. 2002), EPO can normalize the autoregulation of the vessel tone and suppresses expression of the inducible nitric oxide synthase (iNOS) (Schwab et al. 2006a). In order to reduce or prevent secondary damage to the spinal cord effectively, the above mentioned approaches should be applied before or at least immediately after the initial injury, which of course is difficult to realize in a clinical setting. Therefore, the research to attenuate sequelae of secondary injury mechanisms will be very important in future.

1.2.4.2. Promoting plasticity

More than 50% of spinal injuries are neurological incomplete with increasing tendency. Almost all of these patients suffering from SCI still reveal spared rims of white matter at the injury site with spared axons passing through the injury to the distal part of the cord (Kakulas 1999). The promotion of the injury-induced plasticity of spared axons to form collateral sproutings in order to create new functional circuitries may be a promising strategy to improve functional outcome after SCI (Bradbury and McMahon 2006). Because it has been shown that the spinal cord is quite plastic and that this ability for plasticity declines at around 5 of age in humans, it would be favorable to achieve the same level of plasticity that is seen in children in the adult spinal cord (Fawcett 2006, Raineteau and Schwab 2001). Several studies demonstrated that neurotrophic factors can promote spinal cord plasticity and sprouting after injury (Jones et al. 2001). The combination of the neurotrophin NT-3 together with an anti Nogo-A antibody has been shown to result in even stronger sprouting and functional recovery in the partially damaged spinal

cord then the blocking of Nogo-A itself (Schnell et al. 1994, Raineteau et al. 2001, Blochliger et al. 2001, Z'Graggen et al. 1998). Furthermore, inhibitory molecules like CSPGs and tenascin-R in the extracellular matrix seem to be involved in the poor plasticity in adults, since the digestion of the CSPGs by ChABC enhances axonal sprouting and improves functional recovery (Pizzorusso et al. 2002, Bruckner et al. 1993, Bradbury et al. 2002, Fawcett 2006). Hence, promoting plasticity, meaning to change the structure, function and organization of spared neurons e.g. by axonal sprouting in response to an injury, would also be a therapeutic option to improve functional outcome after SCI.

1.2.4.3. Promoting axonal regeneration

Axon regeneration is a product of the balance between the regenerative ability of the axon and the permissiveness of the environment. Altering either of these factors will affect the success or failure of regeneration (Fawcett and Asher 1999). In order to achieve an effective axonal regeneration, a combined therapy that both stimulates axonal growth and neutralizes inhibitors would be needed.

Different types of neuronal growth can contribute to a restoration of input to neurons that lose their normal connections. The most common type is the reactive synaptogenesis. Reactive synaptogenesis refers to the process by which the local growth of afferents terminates near sites of partial denervation. This process also involves the synaptic reinnervation of the partially denervated nerve cell by newly formed (collateral) axonal branches of persisting afferent or local fibres, resulting in the formation of new synaptic contacts. Another type of neuronal growth is termed regenerative sprouting and refers to the process of axonal growth as a response to axonal amputation. In contrast, axonal regeneration occurs as a response of denervation, which leads to the reinnervation of lesioned neurons through the in-growth of new axons and the formation of new connections (Hamori 1990, Matthews, Cotman and Lynch 1976, Steward 1989).

Several intrinsic factors affect the regenerative capacity of axon. As already mentioned above, the axonal regenerative ability declines with age (Chen,

Jhaveri and Schneider 1995, Li, Field and Raisman 1995, Chuckowree, Dickson and Vickers 2004, Nicholls and Saunders 1996) and in addition, some types of axons regenerate better than others (Li et al. 1995). Another important issue is that CNS axons show a greater regenerative response if they are lesioned more proximate (closer to the cell body) compared to distal lesioned axons (Fawcett 2006, Tetzlaff et al. 1994). Furthermore, absent protein synthesis due to the lack of ribosomes in adult CNS axons is probably one reason for their lack of sprouting responses and growth cone regeneration after SCI (Verma et al. 2005) since it has been shown that PNS axons, embryonic axons as well as developing CNS axons contain ribosomes and are capable of protein synthesis (Zheng et al. 2001, Bunge, Bunge and Pappas 1962). The numerous inhibitory molecules present in the environment after a spinal cord lesion also represent important factors that limit effective axonal regeneration. To overcome this hurdle by removing cells that produce inhibitory molecules, preventing or reducing their synthesis, blocking or even degrading inhibitory molecules, would be possible approaches. Moreover, the enhancement of axon growth associated molecules like GAP-43, CAP-23 or L1 could also be a possibility (Becker et al. 2004, Mason, Lieberman and Anderson 2003, Bomze et al. 2001, Buffo et al. 1997).

To regain complete axonal function would be the ultimate intent in SCI research. Whereas it will be difficult to distinguish between functional improvement due to axonal regeneration and functional improvement due to plasticity effects within spared axons, since most SCI models represent incomplete injuries and elicit some spontaneous regain of function by compensation of spared connections.

1.2.4.4 Promoting remyelination

Remyelination of the CNS involves the synthesis of new myelin sheaths by oligodendrocytes to ensheat „naked“ axons in order to regain proper nerve conduction. The promotion of remyelination represents therefore another important strategy to induce recovery after SCI. So far, several strategies have shown to be beneficial.

For example, the inhibition of BMP (bone morphogenetic protein) activity.

BMPs are not only important regulators during CNS development and but they are also up-regulated after injuries to the CNS (Fuller et al. 2007, Setoguchi et al. 2001). Moreover, BMPs promote the astroglial differentiation, while inhibiting the oligodendroglial differentiation of NPC (Bonaguidi et al. 2005, Gross et al. 1996, Nakashima et al. 2001, Samanta and Kessler 2004, Wang et al. 2001). The overexpression of the BMP antagonist Noggin in a SCI-model showed a shift from astrogenic to oligodendrogenic differentiation (Setoguchi et al. 2004). Therefore, blocking the BMP signaling seems to be an attractive therapeutic strategy in order increase remyelination.

The suppression of p57kip2 would be another approach. p57kip2 is a novel intrinsic inhibitor of oligodendroglial differentiation. The protein is, among other cellular processes, involved in cell cycle control (Besson, Dowdy and Roberts 2008). The down-regulation of p57/kip2 has shown to accelerate morphological maturation as well as myelin expression of oligodendrocytes *in vitro*, which makes this a promising approach in order to promote remyelination (Rivera et al. 2010). Another inhibitor of oligodendrogenesis and myelination is LINGO-1 (Leucine rich repeat and Ig domain containing 1) (Mi, Sandrock and Miller 2008). Antagonizing LINGO-1 activity has shown to promote remyelination in demyelination models (Mi et al. 2007, Mi et al. 2009). Thyroid hormone (TH) on the other hand has shown to promote remyelination. It induces the proliferation of OPC and drives their differentiation and maturation towards an oligodendroglial cell type (Ahlgren et al. 1997, Billon et al. 2001, Rodriguez-Pena 1999). Furthermore, TH has been shown to enhance remyelination in an EAE model (Calza et al. 2002, Calza et al. 2005).

As an alternative to targeting single molecules of the oligodendrogenic program, in order to achieve remyelination, an attractive approach might be the use of cells that produce myelinating cells or provide a local microenvironment favoring oligodendrocyte generation. Two strategies might be possible: 1.) the stimulation of endogenous NPC and OPC to produce oligodendrocytes and 2.) the transplantation of cells with the potential to myelinate axons.

1.2.4.5. Cell replacement therapies

The irreversible loss of spinal cord parenchyma including astroglia, oligodendroglia and neurons is one of the key factors responsible for the severe functional impairment of individuals suffering from SCI. Since the adult CNS is not able to intrinsically replace lost spinal cord parenchyma sufficiently, adequate cell replacement strategies are needed in order to promote substantial and structural recovery.

Many cell populations originating from tissues/organs other than the CNS, have been investigated in regard to their regenerative capacity after transplantation into the injured spinal cord. Possible candidates are cells forming PNS type myelin such as Schwann cells and olfactory ensheathing cells (OEC), which have been shown to promote partial structural repair after transplantation into various CNS sites (Weidner et al. 1999a, Blakemore and Crang 1985, Honmou et al. 1996, Sasaki et al. 2004, Akiyama et al. 2004, Lu and Ashwell 2002). OPC/oligodendrocytes also promoted remyelination and functional improvement after transplantation (Windrem et al. 2004, Archer et al. 1997, Brustle et al. 1999). Stem cells represent another cell population, which is quite promising. Depending on the type of stem cell, they are capable of differentiating into relevant glial and neuronal lineages. In general, one has to distinguish between embryonic and somatic stem cells.

Embryonic stem cells, which are derived from the inner cells mass of the blastocyst, are still totipotent until the eight-cell stage, this means that from each of these cells a viable organism can still develop (McDonald et al. 1999). Pluripotent, neuronal stem cells can be isolated from both the embryonic and the adult mammalian CNS of different types of species (Brustle et al. 1999, Gage 2000, McKay 1997, Zompa et al. 1997). Transplanted stem cells are able to specifically adapt to the local CNS environment and to differentiate into the corresponding neuronal or glial subpopulations (Gaiano and Fishell 1998). In contrast, somatic stem cells can be obtained from individual ion organs as soon as organogenesis has occurred, which means from the fetal state through adulthood. Somatic stem cells are usually more restricted in their ability to differentiate into various cell lineages. Therefore, they are considered just to be multipotent. Quite a few somatic stem cells from organs

other than the CNS have been investigated in respect to their capacity to transdifferentiate into neural tissue. In the last few years it turned out that transdifferentiation of somatic stem cells into neural cells is a rare if not non-existing biological phenomenon. Rather, cells of e.g. blood origin, which have been identified to express neural phenotypes after transplantation, underwent cell fusion (Sandner and Weidner 2007).

NPC cells, which have been identified in the adult mammalian nervous system including the spinal cord, represent a promising source to replace scaffold forming astrocytes, remyelinating oligodendrocytes and neurons within the injured spinal cord (Cao, Benton and Whitemore 2002a). NPC at and around the lesion site can be stimulated by the application of appropriate molecules to replace lost spinal cord tissue intrinsically (stimulation of neurogenesis) (Kulbatski et al. 2005, Obermair, Schroter and Thallmair 2008, Thuret et al. 2006). Alternatively, NPC can be isolated from small brain/spinal cord biopsies, propagated in vitro and ultimately transplanted into the injured spinal cord (neural stem cell transplantation). Both approaches have been tested on the preclinical level promoting variable degrees of spinal cord regeneration (Fig.1.5.). A major advantage of adult NPC is that they can be obtained from the patient's own CNS tissue, thus avoiding graft-rejection and ethical concerns as they apply to embryonic stem cells and somatic fetal derived stem cells.

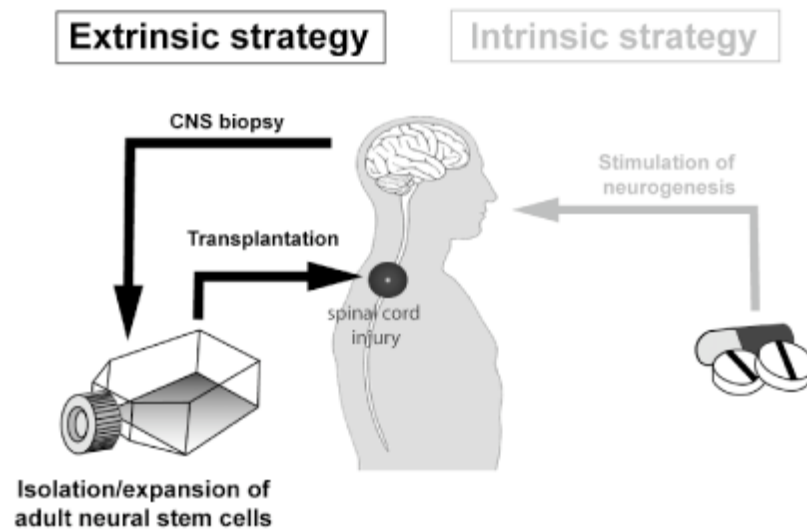


Figure 1.5. Concept of extrinsic (transplantation) and intrinsic (stimulation of endogenous neural cells) replacement strategy

NPC either can be stimulated by the application of appropriate molecules (intrinsic strategy) or they can be isolated from small brain/spinal cord biopsies, propagated in vitro and transplanted into the injured spinal cord (extrinsic strategy).

In the majority of transplantation studies adult NPC were grafted several days to 2 weeks after the actual spinal cord injury. This approach regularly yielded high numbers (about 37%) of surviving neural stem cells (Cao et al. 2001, Enzmann et al. 2005, Hofstetter et al. 2005, Karimi-Abdolrezaee et al. 2006). Nonetheless, transplantation immediately after injury yielded a rather high number of surviving neural stem cells (Vroemen et al. 2003). If transplantation is delayed up to 8 weeks after the injury, the cell survival decreases significantly (Karimi-Abdolrezaee et al. 2006). Not only grafting into the host parenchyma surrounding the spinal cord injury site, but also neural stem cells grafted directly into the lesion cavity/center survived well (Cao et al. 2001, Vroemen et al. 2003). Despite good survival, cystic lesion defects were not replaced by grafted neural stem cells (Vroemen et al. 2003).

Subsequent studies showed that only the combination of neural stem cells with platform providing fibroblasts allowed to maintain neural stem cells within cystic lesion defects (Pfeifer et al. 2004). Following co-grafting of adult neural stem cells with fibroblasts around 43% of the grafted cells took on an astroglial phenotype, whereas roughly 18% differentiated into oligodendrocytes (Pfeifer et al. 2004). Recently, the infusion of various growth

factors including EGF, FGF2 and PDGF-AA at the same time induced a shift of neural stem cells, grafted into the contused rat spinal cord towards, oligodendroglial differentiation (Karimi-Abdolrezaee et al. 2006). Roughly 50% of the grafted neural stem cells differentiated along an oligodendroglial lineage. Further analysis revealed that at least a proportion of grafted cells appeared to remyelinate axons. Remyelination was determined the measurement of the myelination index by electronmicroscopy or by detecting grafted cells, which were MBP-immunoreactive and formed myelin. None of the existing studies investigating the transplantation of undifferentiated adult neural stem cells into the spinal cord observed neuronal differentiation. Only the interference with the differentiation of neural stem cells through growth factor application (Karimi-Abdolrezaee et al. 2006) or transduction with neurogenin-2, which belongs to a group of transcription factors inhibiting gliogenesis (Hofstetter et al. 2005), promoted oligodendroglial differentiation and as a consequence induced enhanced remyelination. Both studies reported functional improvement in various behavioral tasks, however, none of these results can be directly related to the observed remyelination.

The local administration of numerous growth factors has been shown to enhance axonal regrowth and even functional recovery after spinal cord injury. Ideally cells are genetically modified before transplantation to overexpress a given growth factor. Several cell populations, such as fibroblasts and Schwann cells, have been successfully transduced to stably overexpress neurotrophic factors (Weidner et al. 1999a, Grill et al. 1997, Lu, Jones and Tuszynski 2005, Jones et al. 2001). Transplantation of genetically engineered fibroblasts that overexpress neurotrophins are able to stimulate axonal regeneration and to induced locomotor improvement (Grill et al. 1997, Blesch and Tuszynski 2003). Therefore it is desirable to augment the regenerative capacity of adult neural stem cells by transducing them to overexpress growth promoting factors.

Mesenchymal stromal cells (MSC) represents another promising cell type in order to achieve remyelination after SCI. Recently, it has been demonstrated *in vitro* that soluble factors present in the conditioned medium of MSC (MSC-CM) strongly activate and promote the oligodendrogenic fate decision in NPC (Rivera et al. 2006, Rivera et al. 2008), even though the underlying

mechanism and the molecules of this pro-oligodendrogenic effect are still unknown. This differentiation towards the oligodendroglial lineage was demonstrated by a strong increase of cells expressing the oligodendroglial markers GalC and MBP, which was at the expense of astrogenesis, since the number of GFAP-expressing cells was dramatically reduced. Moreover, it has been shown, that the expression of the pro-oligodendrogenic transcription factors Olig1/2 were increased, while the expression of the inhibitor of differentiation Id2 was reduced. These observations indicate that MSC-CM not only promotes oligodendrocyte differentiation and maturation, but also oligodendrocyte fate decision. Furthermore, it has been demonstrated, that transplanted MSC integrate into the host tissue, enhance axonal regeneration and improve functional outcome (Ankeny, McTigue and Jakeman 2004, Hofstetter et al. 2002, Neuhuber et al. 2005, Zurita and Vaquero 2006). MSC further promote oligodendroglial differentiation and remyelination in animal models of stroke and MS (Bai et al. 2007, Lu et al. 2009, Zhang et al. 2009), which make MSC also a promising cell type for cell transplantation approaches in animal models of SC. Several studies demonstrated that grafted MSC (Liu et al. 2008) provide a regenerative host microenvironment by secreting different growth factors, cytokines or bioactive factors that not only regulate proliferation, differentiation and migration, decrease apoptosis or enhance angiogenesis of endogenous stem or progenitor cells, but also inhibit glial scar formation (Caplan 2007, Hung et al. 2007, Munoz et al. 2005, Prockop 2007). Moreover, MSC secrete anti-inflammatory molecules and show immunoregulatory effects by inhibiting T-cell recognition and proliferation (Caplan 2007, Uccelli, Moretta and Pistoia 2008, Uccelli, Pistoia and Moretta 2007, Uccelli et al. 2006). Another advantage of MSC is that they can be easily isolated and rapidly expanded *in vitro* and transplanted back into patients as autografts, avoiding grafts-rejection and ethical concerns, which makes them feasible for the clinical use (Keating 2006, Wright et al. 2008).

1.2.5. Magnetic resonance (MR) imaging for studying spinal cord injury

MR imaging represents the gold standard to visualize the spinal cord in the clinical routine diagnostic setting as well as in clinical research (Weber et al. 2006, Sicard et al. 2006, Thorsen et al. 2003, Guzman et al. 2000, Brandt et al. 2008). MR imaging can be employed to non-invasively analyze pathomorphologic changes in the injured human spinal cord parenchyma, such as hemorrhage, edema and secondary degenerative changes including cystic degeneration and glial scar formation (Sandner et al. 2009, Hadley and Teasdale 1988, Manelfe 1991, Weber et al. 2006). Moreover, MR imaging might provides the facility to predict motor recovery after spinal cord injury (Flanders et al. 1996).

Not only in humans, but also in small animals such as rats or mice MR imaging technology is of high interest for monitoring structural changes in the injured rat spinal cord for several reasons: With MR imaging, structural changes can be assessed over time within identical animals, which not only gives more precise information than correlative histology based studies, but also helps to substantially reduce the numbers of experimental animals. Animal studies allow correlation of MR imaging findings with histology based analysis, a prerequisite to later on plan and interpret MR imaging findings in humans after injury and regenerative therapeutic interventions (e.g. cell-transplantation, biomaterial implantation). In addition, markers (e.g. iron oxide nanoparticles or gadolinium based probes) to identify the location and survival of cell grafts can be validated. Numerous studies in rodents described MR findings in vivo with dedicated experimental imaging systems up to 17.6T field strength combined with surface or implanted coils, which achieve high spatial resolution of morphological changes in the injured spinal cord (Bilgen et al. 2000, Ford et al. 1994, Fraidakis et al. 1998, Fukuoka et al. 1998a, Guizar-Sahagun et al. 1994, Metz et al. 2000, Narayana et al. 2004, Ohta et al. 1999, Weber et al. 2006). However, their usefulness is clearly impaired by their limited availability, rodent size restrictions and potentially higher mortality rate. But various studies of small animal imaging studies have been performed on standard clinical 1.5T and 3T scanners, which are broadly available and

therefore represent a powerful tool to monitor the effects of experimental therapeutic interventions in small animals (Guzman et al. 2000, Linn et al. 2007, Chen et al. 2004a, Fehm et al. 2005).

In animal models, regenerative strategies have been performed in order to promote structural and functional recovery after spinal cord injury. In particular, cell-based therapies have shown promising effects for the treatment of neurological diseases and stroke (Hoehn et al. 2002, Bulte et al. 1999) and in regenerating the injured spinal cord (Grill et al. 1997, Xu et al. 1995b, Pfeifer et al. 2004, Li, Field and Raisman 1997). For the clinical application of these therapeutic approaches, noninvasive imaging techniques that monitor the migration, differentiation processes and the regenerative potential of these cells after grafting, are mandatory.

1.2.6. Introduction to the used animal models of spinal cord injury

1.2.6.1. Cervical dorsal column transection using a Tungsten wire knife device

This lesion model for SCI in rats comprises the bilateral transection of the cervical dorsal columns at the C3 level using a Tungsten wire knife device. The stereotactic guidance of the wire knife device results in highly reproducible lesions, allowing the characterization of local responses and structural alterations after injury. In addition, there is almost no damage to the spinal cord grey matter, and importantly the dura remains intact allowing the injection of cell-suspensions into the lesion with almost no leaking out of the cells. The wire knife lesions cause a rather blunt transection, disrupting the dorsal corticospinal tract (CST), the fasciculus gracilis and the fasciculus cuneatus, with minimal disruption of the vasculature and the surrounding spinal cord cytoarchitecture. Because of this minimal disruption, the infiltration of meningeal fibroblasts is mainly avoided. Over time, the lesion transforms into a cystic cavity, which is restricted to the lesion site (Weidner, Grill and Tuszynski 1999b) (Fig.1.6.).

The wire knife transection model interrupts the crossed component in the ventralmost part of the dorsal funiculus of the CST, which contains 95% of the descending axons and an ipsilateral ventral component containing less than 5% of all CST axons. A large proportion of the overlying dorsal funicular

dorsal sensory ascending projection are also lesioned by this procedure (Weidner et al. 2001). Beyond, the CST also exists of two other minor components, the lateral and dorsolateral components, together constituting less than 2% of CST axons (Vahlsing and Feringa 1980, Joosten et al. 1992, Brosamle and Schwab 1997). The fibers of the CST originate in layer V of the primary motor and sensory cortex (Miller 1987) and control different types of movements through its terminations in the intermediate grey and ventral horn which include direct terminations on motoneurons (Liang et al. 1991). However, the CST is not essential for the control of skilled limb movements such as reaching for and grasping food (Whishaw, Gorny and Sarna 1998), since it has been demonstrated that only combined lesions of both dorsal and ventral CST components eliminated sprouting and impaired function in fine motor tasks such as grasping (Weidner et al. 2001). This specific lesion of the dorsal CST does not result in obvious long-term behavioral deficit. Therefore the described lesion model is suitable to assess morphological changes, in particular the regeneration CST axons, can be examined (Weidner et al. 2001).

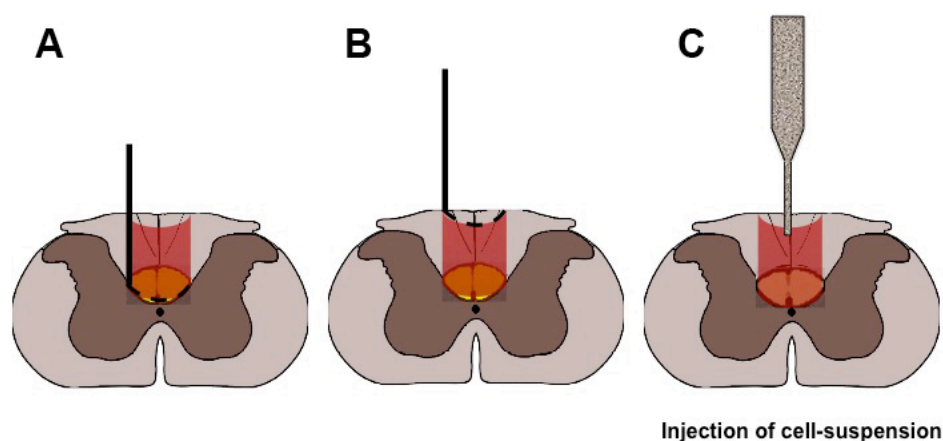


Figure 1.6. Schematic representation of the Cervical dorsal column transection model

The crossed dorsal components of CST axons of rats contain about 95% of the descending axons, whereas an ipsilateral ventral component contains less than 5% of all CST axons. (A) In this SCI model, a dura incision is made and the wire knife device is stereotactically lowered into the spinal cord parenchyma. At the correct depth, the tungsten wire is extruded, forming a wire arch below the dorsal component of the corticospinal projections (yellow). (B) Afterwards, the wire knife device is risen up until the tip of the wire is visible, transecting the dorsal columns bilaterally and interrupting the dorsal component of the corticospinal projections and a portion of the rostrally projecting proprioceptive dorsal sensory pathway. The wire arc was then retracted back into the wire knife device, and the instrument was removed from the cord, thereby leaving the dura intact (C) Directly following the lesion, cell-grafts can be injected under stereotactic guidance into the lesioned area through a pulled glass micropipette.

1.2.6.2. Contusive spinal cord injury using the Infinite Horizon Impactor device

Blunt spinal cord injuries were performed at thoracical level in adult rats using the Infinite Horizon (IH) spinal cord injury device (Precision Systems & Instrumentation, Lexington, KY, USA). This computer-controlled device creates a highly reproducible, well-defined contusion injury by rapidly applying a controlled impact defined in terms of force to the exposed spinal cord. The reproducibility of the injuries is achieved by the application of a force-feedback impounder that measures the employed force to the animal (Scheff et al. 2003). This severe thoracical contusion lesion induces, in contrast to the wire-knife lesion model, lasting behavioural deficits which can be assessed by various behavioral test like the Basso, Beattie, and Bresnahan locomotive rating scale (Basso, Beattie and Bresnahan 1995). In addition, this animal model represents a more clinical related approach and thus allows the pathomorphological comparison between animal model and injured patient.

2. Aim of the Thesis

In incomplete and even complete spinal cord injury a more or less extensive rim of spared descending and ascending axon projections is preserved, which becomes demyelinated and thus fails to mediate neurological function. Based on the recent studies (Rivera et al. 2006, Rivera et al. 2008) showing that MSC derived soluble factors induce oligodendroglial differentiation *in vitro*, the main aim of this study was to determine, whether MSC derived soluble factors will also promote *in vivo* following transplantation into the intact and injured rat spinal cord oligodendroglial differentiation and ultimately remyelination as the structural basis for functional recovery.

Detailed aims of the study were:

1. Determine the potential of MSC-CM to promote oligodendroglial differentiation in SVZ derived NPC *in vitro*.
2. Determine oligodendroglial differentiation in NPC co-seeded with MSC onto hippocampal slice cultures.
3. Determine oligodendroglial differentiation in SVZ derived NPC co-grafted with MSC into the intact spinal cord.
4. Determine oligodendroglial differentiation in pre-differentiated NPC co-grafted with MSC into the intact spinal cord.
5. Determine oligodendroglial differentiation in NPC co-grafted with MSC into the injured rat spinal cord.
6. Determine oligodendroglial differentiation in endogenous NPC after grafting with MSC into injured rat spinal cord.
7. Determine molecular factors underlying the differentiation pattern of NPC grafts *in vivo*.

8. In parallel, as a step towards clinical translation, a routine 3.0T MRI was evaluated regarding its capacity to visualize morphological changes in the contused rat spinal cord as a prerequisite to validate this diagnostic tool for monitoring of structural changes in cell-based therapies in the clinical setting.

3. Material and Methods

3.1. Material

All chemicals were purchased from Merck (Darmstadt, Germany), if not noted otherwise.

3.1.1. Chemicals

3.1.1.1. Cell culture

Chemical	Source
Alpha MEM	Gibco BRL, Germany
Accutase	PAA, Pasching, Austria
B27 supplement	Gibco BRL, Germany
Bromodesoxyuridine (BrdU)	Sigma-Aldrich, Taufkirchen, Germany
Dispase II	Boehringer, Germany
DMEM/F12	Gibco BRL, Germany
DMSO	Sigma-Aldrich, Taufkirchen, Germany
DNase I	Worthington Biochemicals, England
Dulbecco's phosphate buffered saline	Sigma-Aldrich, Taufkirchen, Germany
Epidermal growth factor (EGF)	R&D Systems
Fetal bovine serum (FBS)	PAN, Germany
Fibroblast growth factor (FGF)	R&D Systems
Glucose	Merck, Germany
Hank's Balanced Salt Solution	PAN, Germany
Heparin	Sigma-Aldrich, Taufkirchen, Germany
Laminin	Sigma-Aldrich, Taufkirchen, Germany
L-glutamine	PAN, Germany
Neurobasal Medium (NB)	Gibco BRL, Germany
Papain	Worthington Biochemicals, England
Penicillin/streptomycin	PAN, Germany
Poly-L-ornithine	Sigma-Aldrich, Taufkirchen, Germany
Trypan Blue	Sigma-Aldrich, Taufkirchen, Germany
Trypsin	PAN, Germany

3.1.1.2. Immunodetection

Chemical	Source
4'6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, Taufkirchen, Germany
DAB Peroxidase Substrate Kit	Biozol, Eching, Germany
Donkey serum	PAN, Germany
Gelatin from cold water fish skin	Sigma-Aldrich, Taufkirchen, Germany
Prolong Antifade	Invitrogen GmbH, Germany
Triton X-100	Sigma-Aldrich, Taufkirchen, Germany
Nuclear Fast Red solution	Linaris, Wertheim, Germany

3.1.1.3. Other Chemicals + Kits

Chemical	Company
Ethanol	Baker, Unterschleißheim,
Isoflurane	Baxter
2-Mercaptoethanol	Sigma-Aldrich, Germany
2-Methylbutane (Isopentane)	Fluka
Paraformaldehyde	Sigma-Aldrich, Germany
SYBR® Green JumpStart™ Taq Ready	Sigma-Aldrich, Germany
RNase-free DNase-Set	Qiagen, Hilden, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
Reverse Transcription System	Promega, Mannheim, Germany

3.1.2 Antibodies

Primary antibody	Experimental application	Dil.	Source
ms α APC (mouse anti- Adenomatous polyposis coli)	mature oligodendrocytes	1:500	Calbiochem, Darmstadt, Germany
rt α BrdU (Bromodesoxyuridine)	base analog (proliferation marker)	1:500	AbDSerotec, UK
rb α GalC (Galactocerebroside)	mature oligodendrocyte	1:250	Chemicon, Temecula, CA
rb α GFAP (glial fibrillary acidic protein)	astrocytes	1:1000	Dako, A/S, Glostrup, DK
gt α GFP (green fluorescent protein)		1:500	Rockland, Gilbertsville,
ms α (Map2ab) microtubule associated protein 2ab	mature neurons	1:200	Sigma-Aldrich, Taufkirchen, Germany
ms α MPB (= SMI-94) (myelin basic protein)	mature neurons	1:750	HiSS Diag. Germany

Secondary antibody	Dil.	Source
dk α ms IgG Alexa Fluor 488	1:1000	Molecular Probes, Germany
dk α gt IgG Alexa Fluor 488	1:1000	Molecular Probes, Germany
dk α rb IgG Alexa Fluor 488	1:1000	Molecular Probes, Germany
dk α ms-RHOX (rhodamine X)	1:1000	Dianova, Hamburg, Germany
dk α rb-RHOX	1:1000	Dianova, Hamburg, Germany
dk α rt-RHOX	1:1000	Dianova, Hamburg, Germany
dk α rb-CY5	1:1000	Dianova, Hamburg, Germany
dk α rt-Biotin	1:1000	Dianova, Hamburg, Germany

3.1.3. Buffer and solutions

Reagent	Application	Source
Borate Buffer (0.1M) pH 8.5	- 3.08g boric acid - 450 ml H ₂ O - 5N NaOH to pH 8.5 - fill up to 500ml	Sigma-Aldrich, Germany Sigma-Aldrich, Germany
Cyro Protect Solution (CPS)	- 250 ml Glycerine - 500 ml 0,1 M PO ₄ buffer - 250 ml ethylene glycol	
Donkey serum blocking buffer	- 960 µl TBS - 30 µl donkey serum - 10 µl Triton X-100	Merck, Germany PAN, Germany Sigma, Germany
Fish Skin Gelatin Buffer (FSGB)	- 1000 ml TBS - 10g BSA - 2 ml v Fish Skin Gelatine optional: 1 ml Triton X-100	Sigma, Germany
Phosphate Buffer (PO ₄ : 0.2M)	- 6,35g NaH ₂ PO ₄ x H ₂ O - 41,35g Na ₂ HPO ₄ x 7 H ₂ O - fill up with ddH ₂ O to 1L	Sigma-Aldrich, Taufkirchen, Germany
4% Paraformaldehyde (PFA)	- 40g paraformaldehyde - add 500ml H ₂ O, heat to 70°C - dissolve while stirring - add 1ml 10M NaOH - filter solution and add 500ml 0.2M PO ₄	Sigma-Aldrich, Taufkirchen, Germany
PBS (0.1M)	- 500ml 0.2M Phosphate Buffer - 500ml dH ₂ O - 9g Natriumchloride	
PPD (100ml)	- 0.01% Papain - 0.1% Dispase II - 0.01% Dnase - 149 mg MgSO ₄ *7H ₂ O - in HBSS w/o Ca ₂ ⁺ /Mg ₂ ⁺	Worthington, USA Boehringer Mannheim Worthington, USA PAN, Germany
30% Sucrose	- 300g Sucrose - 400ml 0.2M PO ₄ - 400 ml ddH ₂ O	Sigma-Aldrich, Taufkirchen, Germany
20 x SSC:	- NaCl 175.3 g - NaCitrat x 2 H ₂ O 88.2 g - H ₂ Odest ad 1000 ml Adjust pH 7.0 with 1 N HCl	-
10 x TBS	- Trizma Base 30 g - KCl 2 g - NaCl 80 g - Fill up to 1000ml with H ₂ Odest - Adjust pH 7.4 with 1 N HCl	

3.1.4. RT-PCR primers

All primers were purchased from Invitrogen (Karlsruhe, Germany).

Name	Sequence forward 5' -	Sequence reverse 5' -	Tem p.	Size
Olig1 (Oligodendrocyte transcription factor 1)	GCCCCACCAAGTA CCTGTCTC	GGGACCAGATGC GGGAAC	56°C	109bp
Olig2 (Oligodendrocyte transcription factor 2)	CACAGGAGGGACT GTGTCCT	GGTGCTGGAGGA AGATGACT	56°C	144bp
Id2 (DNA-binding protein inhibitor 2)	TTTCCTCCTACGAG CAGCAT	CCAGTTCCTTGA GCTTGGAG	56°C	160bp
G6PDH (Glucose-6-Phosphat- Dehydrogenase)	CCAGCCTCCACAA GCACCTCAAC	AATTAGCCCCCA CGACCCTCAGTA	58°C	

PCR-Program:

The following temperature profile was used for all the genes analyzed:

1 cycle:	10 min	95°C	activation of polymerase
40 cycles:	30 sec	95°C	denaturation
	1 min	56°C	annealing
	1 min	72°C	elongation
1 cycle:	1 min	95°C	
	30 sec	55°C	
	30 sec	95°C	

The quality of the products was controlled by a Melt curve. An example of RNA quantification is shown in the Results section.

3.1.5. Consumables

Materials	Source
Coverslips and slides	VWR; Mendite
Cell-culture ware	Peske, Aindlingen-Arnhofen, Germany; Corning Costar, Bodenheim, Germany; Sarstedt, Nümbrecht, Germany;
Cell-Culture media	PAN Biotech, Aidenbach, Germany Invitrogen / Gibco, Karlsruhe, Germany
Growth factors	R&D Systems, Germany

3.1.6. Software

Program	Software producer
Adobe Illustrator CS3	Adobe, San Jose, CA, USA
Adobe Photoshop	Adobe, San Jose, CA, USA
EndNote X3	Thomson Reuters, CA, USA
Mircosoft Office for MacOS X (10.5.8)	Redmond, WA, USA
OsiriX for MacOS X	Los Angeles, CA, USA
Prism 4, Version 4.0a for Macintosh	GraphPad Software, San Diego, CA, USA
Spot 3.5.9. for Mac OS	Diagnostic Instruments, Sterling Heights, MI, USA
Leica confocal software	Leica, Wetzlar, Germany
MxPro-Mx3005P software	Stratagene, La Jolla, CA, USA

3.1.7. Equipment and Instruments

Apparatus	Source
Hera Cell incubator	Heraeus, Germany
Hera Safe cell culture hood	Heraeus, Germany
Inverse fluorescence microscope for cell culture	Olympus, Germany
Leica confocal fluorescence microscope (TCS-NT)	Leica, Wetzlar, Germany
Leica fluorescent microscope equipped with a Spot CCD camera model 2.2.1	Leica, Wetzlar, Germany; Diagnostic Instruments, Inc., Sterling Heights, MI, USA
refrigerated table centrifuge	Eppendorf, Hamburg, Germany
table centrifuge mini spin plus	Eppendorf, Hamburg, Germany
Mx3005P Real-Time PCR System	Stratagene, La Jolla, CA, USA

3.2. Methods

3.2.1. Animal subjects

Adult female Fischer 344 rats (Charles River Deutschland GmbH, Sulzfeld, Germany) weighing 160-180g (3-4 months old) were used as donors for the isolation of NPC, MSC and fibroblasts and for all transplantation experiments. Nineteen to 21 days postnatal Wistar rats were used as donors for the hippocampal slice cultures.

All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and institutional guidelines. Animals had *ad libitum* access to food and water throughout the study. All efforts were made to minimize the number of used animals and their suffering.

3.2.2. Preparation and cell culture

3.2.2.1. Preparation of neural progenitor cells (NPC)

For the different cell isolation procedures, adult female Fischer-344 rats or transgenic rats expressing ubiquitously the green fluorescent protein (GFP) reporter (Lois et al. 2002) were deeply anesthetized using a cocktail of ketamine (62.5mg/kg; WDT, Garbsen, Germany), xylazine (3.175mg/kg; WDT, Garbsen, Germany) and acepromazine (0.625mg/kg, Sanofi-Ceva, Düsseldorf, Germany) in 0.9% sterile saline solution and killed by decapitation. Brains and spinal cords were removed and put in 4° C Dulbecco's phosphate buffered saline (DPBS) (PAA Laboratories, Linz, Austria). Overlying meninges and blood vessels were removed. The hippocampus (HC) and ependymal zones, including subependymal and subventricular zones from the lateral wall of the lateral ventricle (SVZ), were aseptically removed. The dissected tissue was transferred to fresh DPBS, washed once, transferred to Petri dishes, and dissociated mechanically. The cell suspension was washed in DPBS to rinse off excess of blood and further digested in PPD solution containing papain (0.01%, Worthington Biochemicals, Lakewood, USA), 0.1% dispase II (Boehringer, Germany), DNase I (0.01%, Worthington Biochemicals) and 12.4 mM MgSO₄, dissolved

in Hank's balanced salt solution (HBSS, PAA Laboratories) for 30 min at 37°C. The cell suspension was triturated every 10 min until the tissue was digested completely. The tissue was centrifuged at 120 x g for 5 min at 4°C and washed three times in Neurobasal (NB) medium (Gibco BRL, Germany) supplemented with B27 (Gibco BRL, Germany), 2 mM L-glutamine (PAN, Germany), 100 U/ml penicillin / 0.1 mg/l streptomycin (PAN, Germany). Cells were resuspended in NB medium supplemented additionally with 2µg/ml heparin (Sigma, Germany), 20 ng/ml FGF-2 (R&D Systems, Germany) and 20 ng/ml EGF (R&D Systems, Germany). Cultures were maintained as neurospheres in uncoated culture flasks at 37°C in a humidified incubator with 5% CO₂. Half of the cell culture medium was changed twice a week, by centrifuging the medium containing the neurospheres at 120 x g for 5 min at 4°C and then removing the supernatant and resuspending the cells in fresh growth medium (Wachs et al. 2003, Hofstetter et al. 2002). Cultures were maintained at 37°C in an incubator with 5% CO₂. Single cells began to form spheres within 5 to 7 days of suspension culture and continued to grow in mass and number during the next weeks. Half of the medium was changed every 3 days. Cell cultures were passaged in weekly intervals. Passaging of the neurospheres was performed as follows: the medium containing the neurospheres was collected in a 15ml centrifuge tube and centrifuged at 120 x g for 5 min at 4°C. The pellet was resuspended in 200µl of AccutaseTM (PAA Laboratories, Linz, Austria) and incubated at 37°C for 10 min. The neurospheres were resuspended in growth medium and triturated. Viable cells were counted by trypan blue exclusion assay in a Neubauer hemocytometer, a total number of 5×10^4 cells/ml was seeded in T75 culture flasks in fresh growth medium.

Neurosphere cultures from passage number 2 to 6 were used throughout this study and termed NPC.

3.2.2.2. Preparation of fibroblasts

Primary cultures of adult Fischer 344 fibroblasts or transgenic rats expressing ubiquitously the GFP reporter (Lois et al. 2002) were generated from skin biopsies and cultivated under standard culture conditions as previously described (Pfeifer et al. 2004, Tuszynski et al. 1994). Briefly, a small skin biopsy from the abdominal region was taken. The biopsy was put into 70% ethanol for a few seconds, washed in HBSS and all fat-tissue was removed. The biopsy was then cut into small pieces (1 x 1 mm) and transferred in culture wells containing Dulbecco's minimal essential medium (DMEM) supplemented with 2 mM L-glutamine (PAN, Germany), 100 U/ml penicillin / 0.1 mg/l streptomycin (PAN, Germany), 3.5 mg/ml Glucose (Merck, Darmstadt, Germany) and 10% fetal calf serum. The medium was changed twice a week. When the cells reached 90% confluence, they were passaged by trypsinization. After counting an aliquot of the resulting single cell suspension in a Neubauer hemocytometer 5×10^4 cells/ml were plated in fresh medium.

3.2.2.3. Preparation of mesenchymal stem cells (MSC)

For isolation of MSC, bone marrow plugs were harvested from femurs and tibias of 2-4 month-old female Fisher-344 rats (Charles River Deutschland GmbH, Germany). Plugs were mechanically dissociated in Minimum Essential Medium alpha Medium (α -MEM) (Gibco Cell Culture, Invitrogen GmbH, Germany) and recovered by centrifugation. Cell pellets were resuspended in α -MEM containing 10% Fetal Bovine Serum (α MEM-10% FBS) (PAN Biotech GmbH, Germany) and seeded at 1×10^6 cells/cm² in a humidified incubator at 37°C with 5% CO₂. After 3 days, the media was changed non-adherent cells were removed. Adherent cells were incubated in fresh α MEM-10%FBS until a confluent layer of cells was reached. These cells were trypsinized using 0.25% Trypsin (Gibco Cell Culture, Invitrogen GmbH, Germany), seeded in α -MEM-10%FBS at 8,000 cells/cm². After 3-5 days of culture, the resulting monolayer of cells, hereafter named rat bone marrow-derived MSC, was trypsinized and frozen or further cultured for experiments. As demonstrated in our previous work, this cell culture preparation is highly enriched in

multipotent MSC with virtually no hematopoietic contamination (Rivera et al. 2006).

3.2.2.4. Preparation and use of Conditioned Media (MSC-CM)

For preparation of MSC-CM, MSC were plated at 12,000 cells/cm² and incubated in α -MEM-10%FBS. After 3 days, MSC-CM was collected and filtered using a 0.22 μ m pore filter. NPC were plated on polyornithine (100 μ g/ml) and laminin (5 μ g/ml)-coated glass coverslips at a density of 10,000 cells per cm² in α -MEM-10% FBS for 12-24 hours. Thereafter, the medium was replaced with MSC-CM or, the cells were kept in control α MED-10% FBS media. After 7 days, cells were fixed for 30 minutes with phosphate-buffered 4% (wt/vol) paraformaldehyde (37°C, pH 7,4) and then processed for immunofluorescence staining.

3.2.2.5. Cell labeling

3.2.2.5.1. Labeling of NPC with BrdU

NPC were incubated with 1 μ M 5-bromo-2'-deoxyuridine (BrdU, Sigma, Germany) in growth medium for 48 h before transplantation. BrdU-incorporation could be demonstrated in more than 95% of NPC by immunohistochemistry (data not shown).

3.2.2.5.2. Labeling of MSC through lentiviral transfection

EGFP-labeled MSC were used in transplantation experiments. We used an HIV-based lentiviral eGFP vector pseudotyped with VSV-G, where eGFP is encoded under the control of the human ubiquitin C promotor (Costa et al. 2008). A total of 10⁷ MSC were transduced in 0.5 ml of medium with 10⁷ infectious viral particles for 1,5 hours at 37°C, washed and seeded. Two days later, the eGFP expressing cells are enriched to approximately 90% by FACS sorting using a FACSAria (Becton Dickinson) (Rivera et al. 2006).

3.2.2.6. Co-cultures of NPC and MSC

Co-cultures of BrdU-labeled NSC and MSC were cultured as described (Rivera et al. 2006). Briefly, MSC (passage 2 to 6) were plated on polyornithine and laminin coated dishes at a density of 2,500-5,000 cells per cm². Twelve to 24 hours later NPC isolated from the SVZ of adult transgenic rats ubiquitously expressing GFP (Lois et al. 2002), were plated over the MSC layer at a density of 10.000 cells per cm² in (αMEM)-10% fetal bovine serum (FBS) and co-cultured for 7 days. Medium was refreshed on the third day. Cells were fixed for 30 minutes with phosphate-buffered 4% (wt/vol) paraformaldehyde (37°C, pH 7.4) and then processed for immunofluorescence staining.

3.2.2.7. NPC pre-differentiation for grafting into the intact spinal cord

NPC derived from the SVZ were pre-differentiated by co-cultivation with MSC. Co-cultures of BrdU-labeled NSC and MSC were cultured as described. Briefly, MSC (passage 2 to 6) were plated on polyornithine and laminin coated dishes at a density of 2,500-5,000 cells per cm². After 16hrs BrdU-labeled NPC were plated over the MSC at a density of 65,000 cells per cm². Cells were incubated in αMEM-10% FBS for 3 days. After this period, co-culture was treated with 0.25% trypsin (Gibco BRL, Germany) and cells were collected for transplantation.

3.2.2.8. NPC pre-differentiation for seeding onto hippocampal slice cultures

NPC (derived from the HC) were pre-differentiated by culturing in MSC-CM. MSC-CM was prepared as described above. Briefly, MSC were plated at 12,000 cells/cm² and incubated in MEM-10% FBS. After 3 days, the conditioned medium was collected and filtered using a 0.22 μm-pore filter. BrdU-labeled NPC were plated on polyornithine (100μg/ml) and laminin (5μg/ml)-coated dishes and incubated for 2 days with MSC-CM. After this period, cells were treated with 0.25% trypsin (Gibco BRL, Germany) and

collected for transplantation. Based on our previous studies, this time frame is sufficient to trigger an oligodendrogenic program in NPC, since i) the presence of MSC-CM for two to three days was sufficient to increase the expression level of pro-oligodendrogenic transcription factors (olig 1/2) and to decrease expression level of the anti- oligodendrogenic factor Id2 (Rivera et al. 2006), and ii) a two day incubation with MSC-CM was sufficient to induce and promote the expression of the oligodendroglial markers Galactocerebroside C (GalC) and myelin basic protein (MBP) and to inhibit expression of the astroglial marker GFAP (Rivera et al. 2008).

3.2.2.9. Preparation of respective cell types for transplantation

A sample of single cell suspension of the NPC was stained with Trypan Blue (Sigma, Germany) and counted in a Neubauer hemocytometer. The remaining single cell suspension was washed twice and resuspended in PBS to yield the desired final concentration. Fibroblasts and MSC were trypsinized and counted in a Neubauer hemocytometer. Cells were washed twice and resuspended in PBS. NPC and MSC/FF suspension were mixed immediate before transplantation.

3.2.3. Immunocytochemistry

Fixed cells were washed in Tris-buffered saline (TBS) (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5), then blocked with a solution composed of TBS, 0.2% fish skin gelatin (Sigma-Aldrich, Germany), 1% bovine serum albumin (BSA, Biomol, Hamburg, Germany) and 0.1% Triton X-100 (Sigma-Aldrich, Germany – only for intracellular antigens (fish gelatin buffer [FGB], AbDSerotec, UK) for 2 h. The same solution was used for the incubation steps with antibodies. Primary antibodies were applied overnight at 4°C. Fluorochrome-conjugated species-specific secondary antibodies were used for immunodetection.

The following antibodies and final concentrations were used: rabbit anti-glial fibrillary acidic protein (GFAP) for astroglia (1:1000; Dako, Denmark A/S, Glostrup, Denmark), rabbit anti-GalC (1:250; Chemicon International, Temecula, CA) and mouse anti-MBP (1:750; SMI-94; Covance, Emeryville, CA) for oligodendrocytes, mouse anti-Microtubule Associated Protein 2ab

(Map2ab) for neurons (1:250; Sigma-Aldrich) and Goat anti-GFP (1:500; Rockland Immunochemicals, Inc., Gilbertsville, PA) was used to detect GFP-positive NPC.

Secondary antibodies: donkey anti-mouse, -rabbit or -goat conjugated with Alexa Fluor 488 (1:1000; Molecular Probes) or rhodamine X (RHOX) (1:500; Dianova, Germany). In case of detergent-sensitive antigens (i.e., GalC), Triton X-100 was omitted from FGB. Nuclear counterstaining was performed with 4', 6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) at 0.25 µg/µl (Sigma-Aldrich). The coverslips were mounted onto glass slides using Prolong-antifade (Invitrogen GmbH, Germany). Epifluorescence observation and photo-documentation were realized using a Leica fluorescent microscope (Leica, Wetzlar, Germany) equipped with a Spot CCD camera model 2.2.1 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

For each culture condition, 10 randomly selected observation fields, containing a total of 500-1,000 cells, were photographed for cell fate analysis. The expression frequency of selected cell type markers was determined for every condition in three independent experiments.

3.2.4. Quantitative RT-PCR

NPC were plated on polyornithine (100 g/ml) and laminin (5 µg/ml)-coated dishes at a density of 30.000 cells per cm² in α -MEM-10% FBS for 12-24 hours. Then, NPC were incubated in either in 1.) MSC-CM, 2.) MSC-CM + 10ng/ml Bone Morphogenic Protein 2 and 4 (BMP 2/4; R & D Systems), 3.) α -MEM-10% FBS + BMP 2 or 4.) α -MEM-10% FBS (as control). Medium change defines day 0. RNA was extracted at day 0, 3, or 7 with the RNeasy kit (QIAGEN GmbH, Hilden, Germany), and cDNA was synthesized using the Reverse Transcription System (Promega). Expression analysis was performed by real-time quantitative PCR with the Mx3005P (Stratagene, La Jolla, CA, USA) with the SYBR[®]Green JumpStart[™] Taq Ready Mix[™] (Sigma-Aldrich, Germany) using specific primers (supplemental online Table 1). As an internal reference, the primer pair for Glucose-6-phosphate dehydrogenase (G6PDH) was used. For quantification, a standard curve was established by amplification of serial dilutions of a mixture of all cDNAs obtained from NPC

cultured under the different conditions. The following temperature profile was used: activation of polymerase: 95°C, 10 minutes; denaturing: 95°C, 30 seconds (40 cycles); annealing: 56°C, 60 seconds; and elongation: 72°C, 60 seconds. The quality of the products was controlled by a Melt curve. Three replicates were run for each cDNA sample with the test and control primers in separate wells of a 96-well plate. The relative quantity of targetgene/referencegene was calculated for each primer. From these results, the ratio of Olig1/Id2 or Olig2/Id2 was calculated.

In parallel, cells were NPC were plated on polyornithine and laminin coated glass coverslips at a density of 10,000 cells per cm² in α MEM-10% FBS for 12-24 hours. Thereafter, the medium was replaced with 1.) MSC-CM, 2.) MSC-CM + BMP 2/4, 3.) α -MEM-10% FBS + BMP 2/4 or 4.) control (α -MEM-10% FBS) media. After 7 days, cells were fixed for 30 minutes with phosphate-buffered 4% (wt/vol) paraformaldehyde (37°C, pH 7,4) and then processed for immunofluorescence staining.

3.2.5. Surgical procedures

For all surgical procedures animals were anesthetized using a cocktail of ketamine, xylazine and acepromazine as described above.

3.2.5.1. Organotypic hippocampal slice cultures and cell transplantation

Preparation and maintenance of organotypic hippocampal slice cultures and cell transplantation were performed as described (Eyupoglu et al. 2005). Briefly, postnatal day 19-21 Wistar rats were decapitated, the brains removed and placed into ice-cold preparation medium. Horizontal 350 μ m sections were prepared on a Leica VT-1000 vibratome (Leica Microsystems, Bensheim, Germany), placed in transwell cell-culture inserts in 6 well plates (Greiner Bioscience, Germany) and cultured as interface cultures (Stoppini, Buchs and Muller 1991) in slice culture medium (Holsken et al. 2006). The slices were cultured in a humidified atmosphere at 35°C. Medium was changed on the first day and every other day thereafter. For stem cell transplantation, a total of 5,000 cells (MSC, NPC or a 1:1 mix of both) were grafted one day after slice culture preparation into different hippocampal

regions (CA region and dentate gyrus) in 0.05 μ l of medium using a Hamilton syringe. Alternatively, 5,000 MSC-CM pre-conditioned (pre-differentiated) BrdU-labeled NPC were transplanted. After 7 days, slices were fixed in 4 % paraformaldehyde for 24 hours and placed in 30% sucrose.

3.2.5.2. Cervical dorsal column transection

Lesions of the dorsal columns were performed using a tungsten wire knife (Kopf Instruments, Tujunga, CA) to transect the dorsal columns bilaterally, thereby interrupting the dorsal component of the corticospinal projection and a portion of the rostrally projecting proprioceptive dorsal sensory pathway. Rats were fixed in a spinal stereotactic unit (Kopf Instruments, Tujunga, CA), skin and muscle were incised, and a laminectomy was performed at C3. A small dura incision was made. For C3 lesions, the tungsten wire knife was stereotaxically positioned 0.6 mm to the left of midline and lowered to a depth of 1.1 mm (for illustration see Fig.1.6.). The tip of the wire knife was extruded from the device, forming a 2.25-mm-wide wire, that was then raised 2 mm to lesion the CST bilaterally. To ensure complete interruption (rather than stretching) of corticospinal axons, a 500- μ m-wide glass pipette was then tightly compressed against the wire loop under microscopic guidance. A large proportion of the overlying dorsal funicular dorsal sensory ascending projection was also lesioned by this procedure. The wire arc was retracted back into the knife device, and the instrument was removed from the cord. This lesion is highly localized and reproducible, permitting precise characterization of local responses to injury and associations of defined axonal populations to the injury milieu (Vroemen et al. 2003, Weidner et al. 1999b, Weidner et al. 2001).

3.2.5.3. Spinal Cord contusion injury

A total of 8 rats received a spinal cord contusion injury at thoracic vertebra (T10) by using the Infinite Horizon (IH) Impactor spinal cord injury device (Precision Systems & instrumentation, Lexington, Ky, USA). This device creates a reliable contusion injury to the exposed spinal cord by rapidly applying a force-defined impact with a stainless steel-tipped impounder. The

instrument does this by employing a stepping motor to apply a controlled impact defined in terms of force. A laminectomy was performed at T10 to expose the dorsal portion of the spinal cord. The exposed vertebral column was stabilized by attaching Adson forceps to the rostral T9 and caudal T11 vertebral bodies. Particular care was taken to align the exposed spinal cord perpendicular to the axis of the Impactor. The 2.5-mm stainless steel impounder tip was lowered to approximately 3–4 mm above the surface of the exposed spinal cord. The contusion injury was finally induced, by applying an impact force of 2 Newton (equal to 200 kilodyne) to the exposed spinal cord at a velocity of 130 mm/s. Overlying muscle layers were sutured and the skin was closed (Weber et al. 2006, Scheff et al. 2003). Postoperatively, animals were kept warm, placed on beds of sawdust, and given manual bladder evacuation twice a day for a period of 10 days as necessary and received intramuscular injections of 10 mg Cotrimoxazol (Ratiopharm, Ulm, Germany) once daily for a period of 10 days. Animals regained automatic neurogenic bladder function after 5-10 days.

3.2.5.4. Cell transplantation into the intact spinal cord

For injection of respective cell types into the intact spinal cord, animals received a partial laminectomy at cervical level C3. Then a total volume of 2µl cell suspension containing a) 1.2×10^5 NPC/µl (**NPC** $n=6$), b) 1.2×10^5 NPC/µl + 0.3×10^5 MSC/µl (**NPC/MS**; $n=6$) or c) 1.2×10^5 NPC/µl + 1×10^5 MSC/µl, which were co-cultured 3 days prior to transplantation (**prediff-NPC/MS**, $n=7$), was injected stereotactically guided (medio-lateral midline, dorso-ventral 0.8mm), using 15-nl per pulse into the spinal cord through a pulled glass micropipette (100µm internal diameter) using a pneumatically driven device (Picospritzer II, General Valve, Fairfield, USA). The micropipette tip remained in place for 25 s before withdrawal. The implantation site was covered with gelfoam (Gelita Tampon; Braun, Germany) before readapting muscular layers and stapling the skin above the lesion.

3.2.5.5. Cell transplantation into the injured spinal cord

Spinal cord lesions were performed as described above. After stereotactically guided transection of the dorsal CST with a tungsten wire knife (David Kopf Instruments, USA) at cervical level C3, a total volume of 2 μ l cells suspension containing a) 0.6×10^5 MSC/ μ l (**MSC**; n=5), b) 1.8×10^5 NPC/ μ l (**NPC** n=5), c) 1.2×10^5 NPC/ μ l + 0.3×10^5 MSC/ μ l (**NPC/MSC**, n=6) or 1.2×10^5 NPC/ μ l + 0.3×10^5 FF/ μ l (**NPC/FF** n=8) was injected directly into the lesion site through a pulled glass micropipette (100 μ m internal diameter) using a Picospritzer II (General Valve, Fairfield, USA). Animals receiving spinal cord lesions without cell transplantation served as controls (**LESION** n=6). For this experiment NPC derived from the SVZ were used.

To investigate the differentiation and survival of endogenous NPC, a total volume of 2 μ l cell suspension containing a) 0.6×10^5 MSC/ μ l (**MSC**; n=6), b) 0.6×10^5 fibroblasts/ μ l (**FF**; n=6) was injected directly into the lesion site. Animals receiving spinal cord lesions without cell transplantation (**Lesion only**, n=6) served as controls. The lesion/implantation site was covered with gelfoam before readapting muscular layers and stapling the skin above the lesion.

3.2.5.6. BrdU-Injection

A short course of BrdU-injection (50 mg/kg) was done intraperitoneally (i.p.) starting right after the surgery until day 3 post-op. Animals were perfused on day 3 to study endogenous cell proliferation (early time-point). For investigations on survival of newborn cells derived from endogenous NPC, BrdU (50mg/kg) was administered i.p. from day 3-10 before the animals were sacrificed on day 28 (late time-point) (for illustration see Fig.4.10.).

3.2.6. Histology

Animals were transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cords were dissected, post fixed overnight and cyroprotected in 30% sucrose. Cervical spinal cords of unlesioned animals or a half of the animals, which received a contusion injury, were cut into sagittal 35µm thick cryostat sections (CM 3000, Leica, Bensheim) and processed for immunohistochemistry. Every seventh section was taken for Nissl staining to determine the injection site. A 3mm long part of the cervical spinal cords containing the lesioned area from animals, which underwent dorsal column transection or spinal cord contusion injury, were cut into coronal 40 µm thick cryostat sections. Three sections (lesion center [LC], rostral and caudal) with a distance of 1120µm between each other were processed for immunohistochemistry. Every seventh section was taken for Nissl staining to determine the lesion/injection site and to assess the overall neuropathological changes (tissue loss, hemorrhage, cyst formation). (Fig.3.1.). Prussian blue staining was employed to identify hemosiderin deposits for the animals, which received a contusion injury.

A 9 mm long part of the cervical spinal cords containing the lesioned area from animals for the endogenous study, was cut into coronal 40 µm thick cryostat sections. Three sections (lesion center [LC], rostral [RO] and caudal [CA]) with a distance of 3 mm between each other were processed for BrdU-DAB staining and immunohistochemistry. Every seventh section was collected for Nissl staining to exactly determine the lesion/injection site. Sections were stored in 25% glycerol, 25% ethylene glycol and 50% 0,1 M sodium phosphate solution at 4°C. (Fig.3.2.)

3.2.6.1. Nissl Staining

The Nissl staining is used in particular to display important structural features of neurons. This is achieved by various basic dyes (e.g. thionine, or cresyl violet) which bind to basophile (negatively charged) compounds like RNA and DNA and therefore stain the nucleus and ribosomes blue or violet. Because cell organells of nerve tissue exist only in their soma and dendrites of neurons, but not in the axon, only the cell bodies will be stained. The cryostat

cut sections were put on gelatin-coated coverslips. The coverslips were then hydrated in a downward ethanol series starting with a 1:2 chloroform/ethanol mixture. The coverslips were incubated in each case for 2 min in 100%, 95%, 70% and 40% ethanol and then for 5 min in H_2O_{dest} . The staining was done by incubation the coverslips into a thionine-solution for 30 sec or longer, depending on the sections. Afterwards, the coverslips were dipped into H_2O_{dest} several times before they were incubated for 2 min each time in an ascending ethanol series (40%, 70%, 80%, 95%) in order to dehydrate the sections again. After incubating the coverslips for about 5 min in NeoClear, the sections were mounted wet in NeoMount.

3.2.6.2. Prussian Blue Staining to detect iron

The cryostat cut sections were put on gelatin-coated coverslips. The coverslips were incubated for 15 min in a solution composed of 4% potassium ferrocyanide ($K_4Fe(CN)_6$) and 4% HCl. After rinsing the sections several times in H_2O_{dest} , they were counterstained with Nuclear Fast red solution (Linaris, Wertheim) for 5 min. After 5x washing with H_2O_{dest} , the sections were dehydrated through a isopropanol series. After incubating the coverslips for about 5 min in NeoClear, the sections were mounted wet in NeoMount.

3.2.7. Immunohistochemistry

3.2.7.1. DAB Staining

Diaminobenzidine (DAB) labelings were obtained as follows: Free-floating sections were treated with 0.6% H_2O_2 in Tris-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 min. For immunohistological detection of the incorporated BrdU, pre-treatment of tissues was performed as followed: after rinsing in TBS, sections were incubated for 30 min in 0.6% H_2O_2 and rinsed again in TBS. Afterwards, the sections were incubated for 1h in 50% formamide/2xSSC (0.3M NaCl, 0.03M sodium citrate) at 65°C. Sections were rinsed in 2xSSC, incubated for 30 min in 2M HCl at 37°C and rinsed for 10 min on 0.1M boric acid (pH 8.5). Following extensive washes in TBS, sections were blocked with a solution composed of TBS, 0.2% fish skin gelatin (Sigma-Aldrich, Germany), 1% bovine serum albumin (BSA, Biomol, Hamburg,

Germany) and 0.1% Triton X-100 (Sigma-Aldrich, Germany – only for intracellular antigens) (fish gelatin buffer (FGB)) for 1 h. This buffer was also used during the incubation with the antibody rat anti-BrdU (1:500). The primary antibody was applied overnight at 4°C. For chromogenic immunodetection, sections were washed extensively and further incubated with a biotin-conjugated species-specific secondary antibody, donkey anti-rat biotin conjugated (1:500; Dianova, Germany), followed by a peroxidase–avidin complex solution from the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, USA). Sections were developed in TBS containing 0.25mg/ml 3, 3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, USA), 0.01% (v/v) H₂O₂, and 0.04% (w/v) NiCl₂. Sections were mounted on gelatin-coated slides and coverslipped with Neo-Mount (Merck, Darmstadt, Germany).

Sections were photographed using a Leica DMR microscope (Leica, Wetzlar, Germany) equipped with a Spot™ CCD digital camera model 2.2.1 (Diagnostic Instrument Inc, Sterling Heights, USA) and epifluorescence observation was performed on a confocal scanning laser microscope (Leica TCS-NT, Wetzlar, Germany).

3.2.7.2. Immunfluorescence Labeling

Double/triple labeling immunofluorescence techniques were performed with free-floating sections to assess the NPC differentiation pattern *in vivo*. Sections were washed in TBS, then blocked with a solution composed of TBS + 3% donkey serum + 0.1% Triton-X100 (only for intracellular antigens) for 1h, and incubated with primary antibodies in the same solution overnight at 4°C on a rotating platform.

The following primary antibodies were used: rat anti-BrdU (1:500) for grafted NPC, rabbit anti- GFAP for astroglia (1:1000), mouse anti- Adenomatous polyposis coli for oligodendrocytes (APC; 1:500, Calbiochem, Darmstadt, Germany). For detection of BrdU-prelabeled nuclei of adult NPC the following DNA denaturation steps preceded the incubation with rat anti-BrdU antibody: after rinsing in TBS, sections were incubated for 1h in 50% formamide/2xSSC (0.3M NaCl, 0.03M sodium citrate) at 65°C. Sections were rinsed in 2xSSC, incubated for 30 min in 2M HCl at 37°C and rinsed for 10 min in 0.1M boric

acid (pH 8.5). After rinsing in TBS, in rat anti-BrdU in TBS / 3% donkey serum / 0.1% Triton-X100 as described above. The following day, sections were rinsed and incubated with fluorophor rhodamine-X (1:1000; Dianova, Germany), fluorescein (1:1000; Molecular Probes), or Cy5 (1:1000; Dianova, Germany) conjugated donkey secondary antibodies for 2h. After a final rinsing step in TBS, sections were mounted onto glass slides and coverslipped with Prolong Antifade (Invitrogen GmbH, Karlsruhe, Germany).

3.2.8. Immunohistochemical analysis

3.2.8.1. Immunohistochemical analysis of DAB stained sections

Quantitative analysis of BrdU-DAB labeled cells was performed using a 40× objective lens of a light microscope (Leica, Wetzlar, Germany).

For the study in the intact animal, all BrdU-positive cells were counted within the section containing the most cells. Graphs are expressed as the total number of cells detected.

For the endogenous study, the number of BrdU-DAB positive cells was counted in three sections from different levels of spinal cord: one in the lesion center (LC), one 3 mm rostral (RO) and another one 3 mm caudal (CA) to the lesion center. Within each section one field of view was quantified in up to seven different regions (for illustration see Fig.3.2.): 1.) central canal (CC), 2.) grey matter / white matter transition zone (GM/WM), 3.) corticospinal tract (CST), 4.) ascending tract (AT), 5.) grey matter (GM), 6.) lateral column white matter (LCWM) and 7.) dorsal root entry zone (DREZ). Graphs are expressed as the total number of cells detected in all seven regions and in all 3 sections.

3.2.8.2. Immunohistochemical analysis of immunofluorescence-labeled sections

Analysis of immunofluorescence-labeled sections was performed using confocal fluorescence microscopy (Leica TCS-NT) equipped with a 40x PL APO oil objective (1.25 numeric aperture). Co-localization of BrdU pre-labeled NPC with the individual differentiation marker was determined by analyzing between 30-35 adjacent optical sections through the z-axis of a 40 µm thick coronal sections. Co-localization was confirmed, once the differentiation marker was spatially associated with BrdU nuclear labeling through

subsequent optical sections in the z-axis. The *in vivo* differentiation pattern for the animals that underwent dorsal column transection, was quantified in one section rostral and one caudal to the lesion by analyzing two different regions (for illustration see Fig.3.1.): 1. dorsal column white matter (DCWM) and 2. dorsal horn grey matter (GM), or directly in the lesion center/graft (LC) for each differentiation marker.

In unlesioned animals, one sagittal section per differentiation marker was quantified by analyzing one field of view in three different regions: 1.) directly at the injection site in the dorsal column white matter (ISWM), 2.) caudal to the injection site in the dorsal column white matter (WM) and 3.) in the grey matter (GM) just ventral to the injection site.

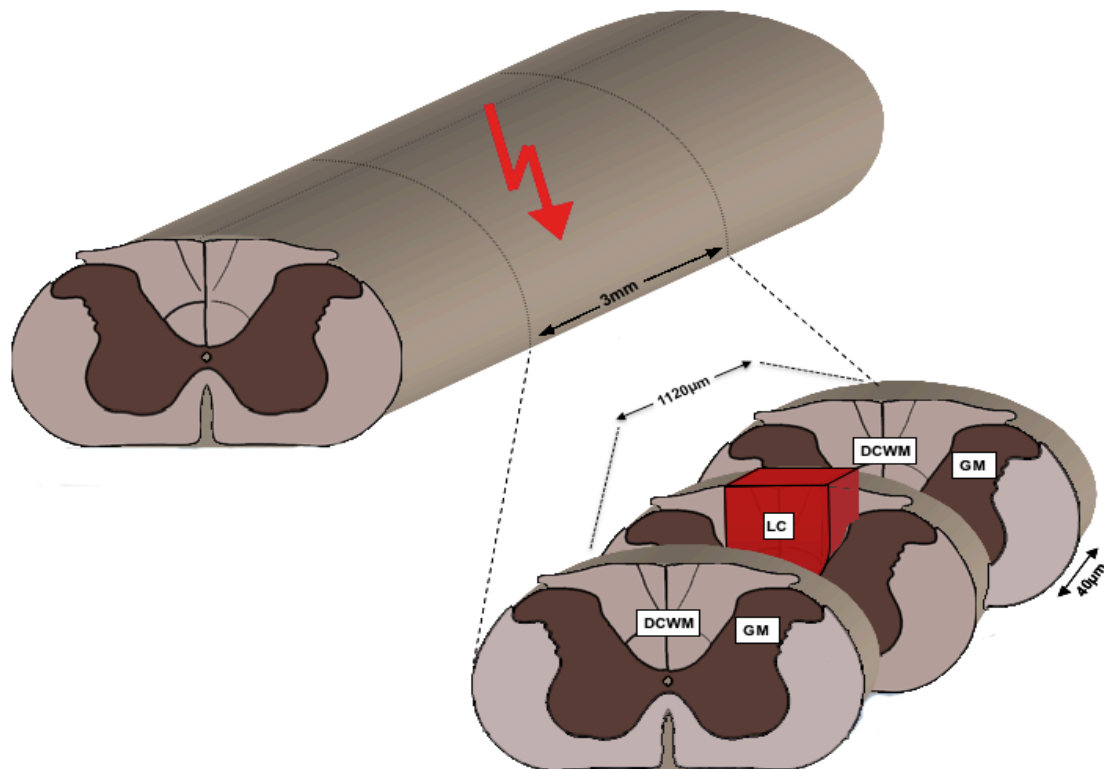


Figure 3.1. Schematic representation of the morphological analysis of grafted NPC the injured spinal cord

A 3 mm long piece of the spinal cord was cut into 40 µm thick coronal sections. For quantification for each differentiation marker, one section rostral and one caudal to the lesion center (LC) was analyzed in two different regions: 1. dorsal column white matter (DCWM) and 2. dorsal horn grey matter (GM), or directly in the lesion center (LC). Each of the analyzed sections had a space of 1120 µm from each other. Red arrow indicates the lesion site.

The *in vivo* differentiation pattern of endogenous spinal cord NPC was quantified in three sections from different levels of spinal cord: one in the lesion center (LC), one 3 mm rostral (RO) and another one 3 mm caudal (CA)

to the lesion center. Each section (LC, RO, CA) was quantified by analyzing the differentiation markers in one field of view in four different regions (for illustration see Fig. 3.2.; 1.) central canal (CC), 2.) grey matter / dorsal column white matter transition zone (GM/WM), 3.) corticospinal tract (CST) and 4.) ascending tract (AT).

The total number of BrdU positive cells within each section was counted and correlated to the number of BrdU positive cells co-localizing with the respective differentiation marker (GFAP, APC). Because APC immunostaining is not restricted to oligodendrocytes, and a subset of astrocytes becomes also labeled with APC (McTigue, Wei and Stokes 2001, Bhat et al. 1996), cells showing the markers BrdU and APC in absence of a GFAP-signal were counted as oligodendroglia, cells expressing BrdU and GFAP in parallel were counted as astroglia.

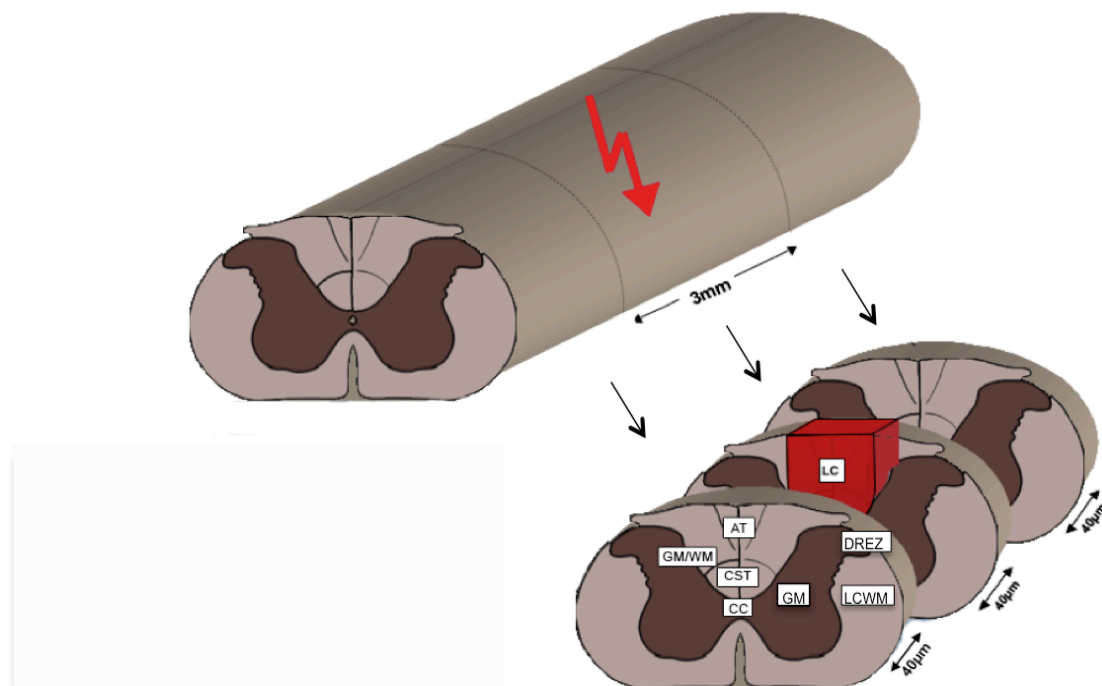


Figure 3.2. Schematic representation of the morphological analysis of endogenous NPC in the injured spinal cord

A 9 mm long piece of the spinal cord was cut into 40 µm thick coronal sections. Three sections (lesion center [LC], rostral [RO] and caudal [CA]) with a distance of 3 mm between each other were quantified. Within each section one field of view was quantified in up to seven different regions: 1.) central canal (CC), 2.) grey matter / white matter transition zone (GM/WM), 3.) corticospinal tract (CST), 4.) ascending tract (AT), 5.) grey matter (GM), 6.) lateral column white matter (LCWM) and 7.) dorsal root entry zone (DREZ). For quantification of the BrdU-DAB staining, all seven regions were analyzed, and for the fluorescence staining, only the first four regions (CC, GM/WM, CST and AT) were analyzed. Red arrow indicates the lesion site.

3.2.9. MR scanner

All MR imaging experiments were conducted on a 3T clinical dedicated head MR scanner, Siemens Magnetom Allegra (Siemens Medical Solutions, Germany). The Magnetom Allegra system was equipped with four independent receiver channels and gradients achieving peak gradient amplitudes of 40mT/m per axis and a minimum slew rate of 400 T/m/s. Only standard Siemens product sequences were used with modifications of the imaging protocols to adapt to small animal imaging. Besides the standard hardware of the MR scanner a dedicated four-channel rat spine phased array coil (RAPID Biomedical GmbH, Germany) was used. The operating frequency of the spine array is 123.2 MHz. It consists of four equivalent rectangular surface coil elements with a size of 20 mm x 30 mm each. They are orientated along z-direction for best coverage of the spinal cord. The array elements are fixed tuned and matched to 50 Ω for loading an average sized rat. An active decoupling mechanism switches the array coil off-resonant during RF transmission. The body coil of the MR system is used for sample excitation. In addition low noise preamplifiers assure the same high signal to noise ratio for a wide range of load variations.

3.2.9.1. MR imaging

Anesthesia was induced by using an inhalation mixture of 5% isoflurane in 30 % O₂ and air mixture and then maintained by using 1.5% of isoflurane. A total of eight spinal cord contused rats underwent MR imaging on day one post injury (MR imaging on day 1; n=4). Out of these, four animals received follow-up MR imaging on day 43 post injury (MR imaging on day 1 and 43; n=4). Normal spine gray and white matter has been characterized on intact female Fischer 344 rats (n=4). The rats were placed in supine position over the rectangular surface of the coil housing. No corrections for motion artifacts arising out of spontaneous respiration or from blood flow were applied. 2D coronal and sagittal images of the spine were acquired using the T2 weighted turbo spin echo (TSE) sequences with repetition time (TR) of 2340 ms, echo time (TE) of 113 ms, echo train length of 6, image matrix 128 x 128 and field of view (FOV) 2.5 cm. The acquired images had an in-plane resolution of 200

μm with a section thickness of 400 μm and no gap between sections. The total acquisition time with 3 averages and 11 slices in 2 concatenations was 5 minutes and 35 seconds. T1 weighted spin-echo (SE) were acquired with the following sequence parameters; TR of 400 ms, TE of 19 ms, image matrix 64 x 128, and a FOV of 1.25 cm x 2.5 cm. The acquired images had an in plane resolution of 200 μm with a section thickness of 1 mm. The total acquisition time with 6 averages and 15 slices in 2 concatenations was 10 minutes and 6 seconds.

Proton density (PD)- weighted TSE images were acquired with a TR of 2500 msec, TE of 16 msec, image matrix 128 x 128, and an FOV of 2.5 x 2.5 cm. The images had an in-plane resolution of 200 μm with a section thickness of 1 mm. The total acquisition time with four averages and 15 slices was 6 minutes and 14 seconds. OsiriX software (Version v.2.6fc1 32-bit) was used for image processing and 3D rendering.

3.2.10. Statistical analysis

Statistical analysis was performed using parametric two-tailed *t* test or for the slice culture experiments one-tailed Mann Whitney *t* test, two-way ANOVA for the RT-PCR data and one-way analysis of variances (ANOVA) followed by Tukey's Multiple Comparison test for multiple group comparisons (*, $p < .05$; **, $p < .01$; *** $< .001$). Averages are expressed with their standard error of the mean (SEM) and or for the slice culture experiments with their standard deviations (SD). Statistical analysis were performed using PRISM4 software (GrapPad, San Diego, CA, USA).

4. Results

4.1. MSC promote oligodendroglial differentiation in SVZ derived NPC *in vitro*

A recently published study by our group focusing on the oligodendroglial determination and differentiation demonstrated that MSC secrete one or more yet unidentified factors, which strongly promote oligodendroglial differentiation of hippocampal adult NPC in co-culture conditions *in vitro*, whereas the astrogenic commitment of NPC is inhibited (Rivera et al. 2006). To confirm that the MSC derived oligodendrogenic effect is not restricted to hippocampal derived NPC and therefore is not region specific, SVZ derived NPC were co-cultured with MSC for 7 days. SVZ derived NPC grown in α -MEM-10%FBS served as control.

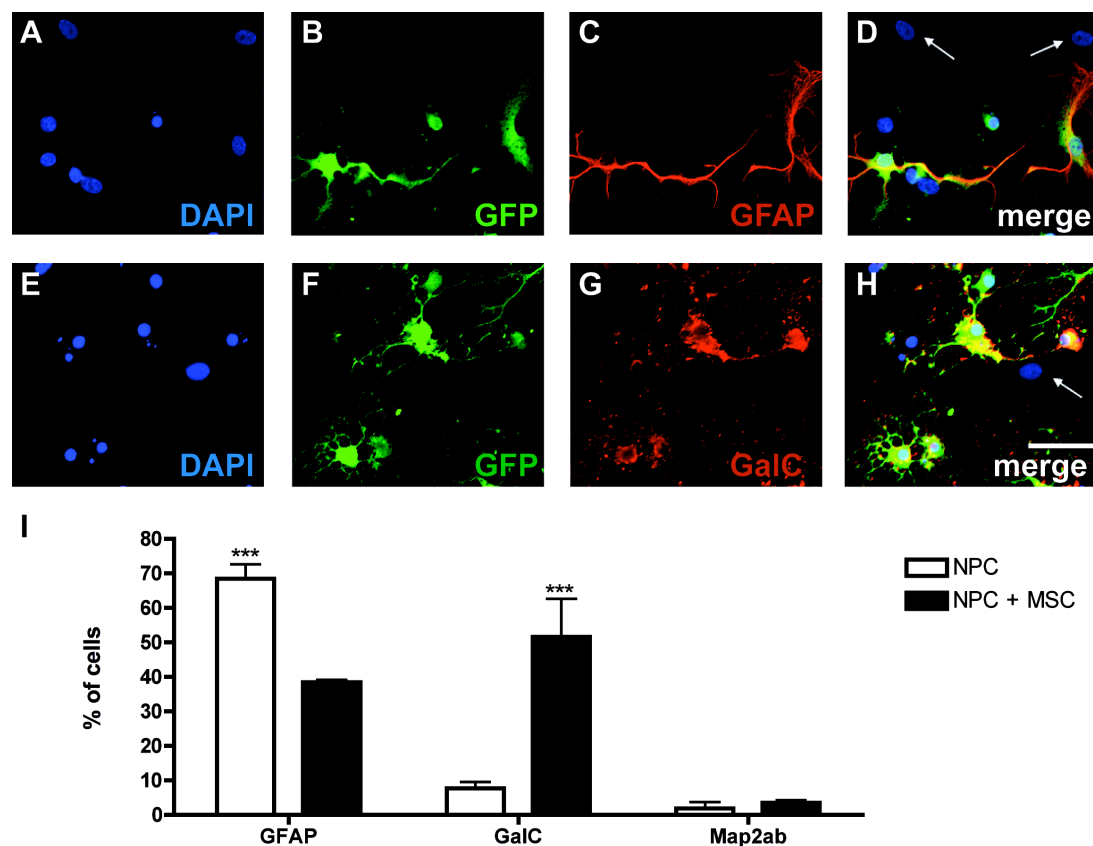


Figure 4.1. Cocultures of MSC and NPC promote oligodendrogenesis of SVZ derived NPC *in vitro*

MSC and NPC (derived from GFP-expressing rats) were co-cultured for 7 days. NPC display immunoreactivity for (C) the astroglial marker GFAP and (G) the oligodendroglial marker GalC. Differentiation markers are shown in red, GFP in green, DAPI nuclear counterstain is shown in blue; merged pictures are on the right. Note that expression of the neural markers is restricted to NPC-derived cells. (I) Quantitative analysis of the expression of the differentiation pattern of adult NPC in control and in co-cultures (NPC + MSC) conditions. The percentage of GFP-positive cells expressing GFAP, GalC or Map2ab was determined. Five hundred to 1,000 GFP-positive cells were analyzed in randomly chosen fields. Experiments were done in triplicate. Data represent standard error of the mean (SEM). Non-parametrical one-tailed Mann Whitney *t* test was used for statistical analysis. ** $p < 0.01$; *** $p < 0.001$. Scale bar: 25 μ m.

As expected, SVZ derived NPC co-cultured with MSC expressed all three mature neural lineages, MAP2ab (neurons), GFAP (astroglia) and GalC (oligodendroglia). Consistent with previous experiments, the number of NPC expressing GalC was significantly increased in co-cultures with MSC (GalC: $48.6\% \pm 5.7$) compared to NPC alone (GalC: $7.5\% \pm 2.7$). As a consequence, the proportion of GFAP-expressing NPC declined in co-culture conditions ($39.1\% \pm 6.3$) compared to NPC cultured alone ($69.5\% \pm 5.4$) (Fig.4.1.). In contrast, the percentage of cell expressing the neuronal marker Map2ab did not significantly differ between both culture conditions (co-culture: $2.8\% \pm 0.8$; control: $1.9\% \pm 1.0$).

4.2. MSC-CM promotes oligodendroglial differentiation of SVZ derived NPC *in vitro*

Incubation of NPC with MSC-derived supernatants yielded similar results: a significantly higher proportion of GalC and MBP expressing cells compared to control cultures (GalC: $39.2 \pm 0.5\%$ versus $3.1 \pm 0.6\%$; MBP: $31 \pm 3\%$ versus $1.7 \pm 0.3\%$), a decrease in the number of GFAP-expressing cells ($26.8 \pm 2.8\%$ versus $41.9 \pm 4.8\%$) and no significant changes in Map2ab expression ($0.8 \pm 0.4\%$ versus $1.1 \pm 0.5\%$) (Fig.4.2.). Taken together, oligodendrogenic effects of MSC co-cultivation and MSC-CM on SVZ derived NPC is comparable to previously described effects on hippocampal NPC.

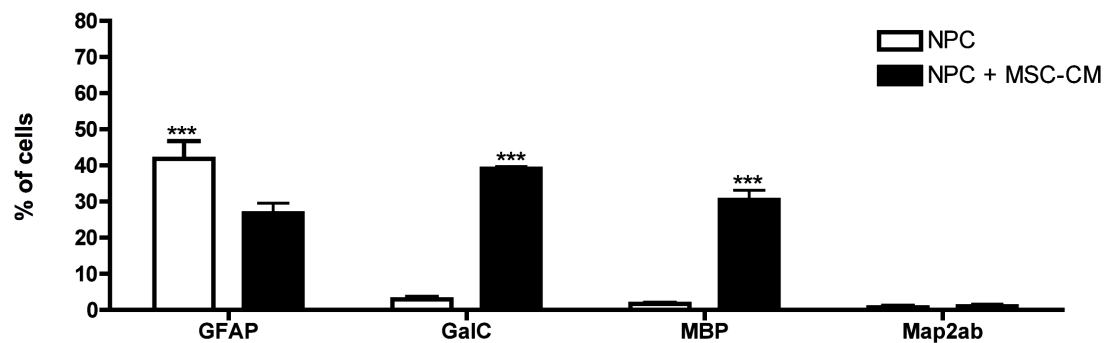


Figure 4.2. MSC soluble factors induce the expression of oligodendrocyte markers in SVZ-derived NPC *in vitro*

NPC were incubated for 7 days in control medium (α -MEM-10% FBS) and in MSC-CM. Quantitative analysis of the expression of neural differentiation markers in control conditions and with MSC-CM (NPC + MSC-CM). The percentage of cells expressing GFAP, MBP, GalC or Map2ab was determined. Five hundred to 1,000 GFP-positive cells were analyzed in randomly chosen fields. Experiments were done in triplicate. Data represent standard error of the mean (SEM). Non-parametrical one-tailed Mann Whitney *t* test was used for statistical analysis; ** $p < 0.01$; *** $p < 0.001$. Abbreviations: MBP: Myelin basic protein.

4.3. Oligodendrogenic effect of MSC-CM and FF-CM on NPC cultures derived from different regions

To test whether this pro-oligodendroglial effect is specific for NPC derived from a particular region of the CNS, NPC derived from subventricular zone (SVZ), hippocampus (HC) and spinal cord (SC) were cultured in MSC-CM for one week and subsequently analyzed for the differentiation markers GFAP, MBP and Map2ab. NPC grown in α -MEM-10%FBS served as control. Furthermore, based on previous findings that fibroblasts promote a rather glial differentiation in co-cultured NPC and that the co-grafting of NPC with fibroblasts following spinal cord injury not only replaces the cystic lesion defect but also leads to an enhanced axonal regeneration (Pfeifer et al. 2004), two more groups were added to this experimental approach: NPC cultured with conditioned medium derived from fibroblasts (FF-CM) and NPC cultured with a mixture of conditioned medium of MSC and fibroblasts (MSC-CM +FF-CM).

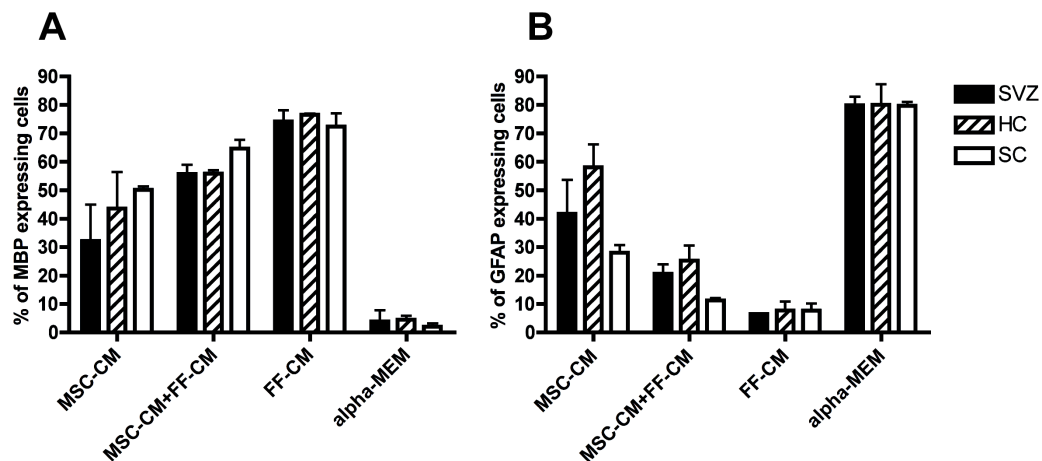


Figure 4.3. The origin of the NPC does not influence their differentiation potential *in vitro*

NPC were incubated for 7 days in MSC-CM, FF-CM and in MSC-CM+FF-CM or in control medium (α -MEM-10% FBS). Quantitative analysis of the expression of neural differentiation markers under the different culture conditions analyzed. The percentage of cells expressing (A) MBP or (B) GFAP was determined. Five hundred to 1,000 GFP-positive cells were analyzed in randomly chosen fields. Experiments were done in triplicate. Data represent standard error of the mean (SEM). One-Way ANOVA was used for statistical analysis.

By comparing the different culture conditions in relation to the distinct origins of the NPC, no significant alterations could be detected for any of the tested markers under the four culture conditions (MSC-CM, MSC-CM+FF-CM, FF-CM, α -MEM-10%FBS) (Fig. 4.3.).

On the other hand, the comparison of the different culture conditions for the glial markers GFAP and MBP within the three NPC-groups (SVZ, HC, SC) revealed significant differences. Significantly less cells expressed the astroglial marker GFAP in SVZ derived NPC incubated with MSC-CM (MSC-CM: $44.6\% \pm 7.4$; MSC-CM+FF-CM: $20.7\% \pm 3.3$) compared to control conditions ($80.6\% \pm 1.8$). Moreover, this reduction was even stronger in NPC incubated with FF-CM ($6.5\% \pm 0.1$). In contrast, the amount of MBP positive NPC was significantly increased FF-CM cultures (FF-CM: $74.2\% \pm 4.0$; MSC-CM+FF-CM: $55.7\% \pm 3.2$) compared to NPC treated with MSC-CM ($27.9\% \pm 8.6$) or α -MEM-10%FBS ($2.9\% \pm 2.5$). The proportion of Map2ab (for neurons) expressing cells was in all condition very low or not detectable (data not shown).

Results

Similar results were obtained for NPC derived from the hippocampus. A significantly higher proportion of NPC expressed the marker MBP in all cultures treated with conditioned media (MSC-CM: 43.6% \pm 12.8; FF-CM: 76.6% \pm 0.5; MSC-CM+FF-CM: 55.8% \pm 1.3) compared to the control (4.5% \pm 1.4). Whereas significantly fewer NPC were GFAP positive after the incubation with FF-CM (FF-CM: 7.8% \pm 3.2; MSC-CM+FF-CM: 25.2% \pm 5.4) compared to MSC-CM (58.1% \pm 8.1) and control conditions (80.1% \pm 7.2).

By comparing the expression of GFAP and MBP under different culture conditions for SC-derived NPC, the just described results were even more prominent. The proportion of MBP positive cells was again significantly increased in all culture conditions (MSC-CM: 50.1% \pm 0.9; FF-CM: 73.4% \pm 3.4; MSC-CM+FF-CM: 54.5% \pm 10.4) compared to the control (2.4% \pm 0.9). Furthermore, differences between the groups incubated with either MSC-CM or FF-CM were also significant. The percentage of GFAP positive NPC was significantly decreased in all conditions (MSC-CM: 27.5% \pm 2.0; FF-CM: 10.7% \pm 0.9; MSC-CM+FF-CM: 12.8% \pm 1.6) compared to control conditions (78.0% \pm 2.0). Under FF-CM containing conditions the number of GFAP positive cells was reduced significantly compared to cells incubated with MSC-CM.

Overall the highest percentage of MBP positive cells, as well as the lowest percentage of GFAP positive cells was always found in the group treated with FF-CM, independent of the region NPC were derived from (SVZ, HC or SC). Furthermore, no additive effect in terms of MBP- or GFAP-expression could be detected in cultures incubated with both, MSC-CM and FF-CM.

In summary, these data show that the incubation of NPC with MSC-CM promotes their differentiation into MBP expressing oligodendrocytes in expense of astroglial differentiation, independent of the origin of the NPC. Moreover, this pro-oligodendrogenic effect is not specific to MSC-CM. FF-CM exerts an even stronger pro-oligodendrogenic effect on NPC (Fig.4.3.).

4.4. Oligodendrogenic effect of MSC on NPC seeded on organotypic hippocampal slice cultures

Previous studies demonstrated that MSC as well as MSC-derived soluble factors induce oligodendrogenesis in expense of astrogenesis and promote oligodendroglial differentiation/maturation in adult rat HC-derived NPC *in vitro* (Rivera et al. 2006, Rivera et al. 2008). As mentioned above, the same results were observed for adult rat NPC derived from the SVZ and the SC. To see if this pro-oligodendrogenic activity is maintained after co-seeding of NPC with MSC into a CNS-organotypic environment, hippocampal slice cultures were used as a target tissue. Organotypic hippocampal slice cultures have the advantage that they are easy to obtain and retain the three-dimensional cyto-architecture of the tissue of origin. They therefore preserve most of the synaptic and anatomical organization of the neuronal circuitry and show functional characteristics similar to those found *in vivo* (Gahwiler et al. 1997, Costa et al. 2008, Miyata et al. 2001, Laskowski et al. 2005, Stoppini et al. 1991).

4.5. Limited survival of NPC pre-differentiated towards oligodendroglia after seeding onto hippocampal slice cultures

The first question was whether adult NPC pre-differentiated towards an oligodendroglial lineage by MSC-CM could survive, integrate and generate oligodendrocytes after seeding onto hippocampal slice cultures. In order to identify NPC, cells were incubated with BrdU prior to seeding (Rivera et al. 2006). Pre-labeled NPC were incubated with MSC-CM for two days prior to seeding on hippocampal slices (Fig. 4.4.A). However, 7 days after seeding, no BrdU-labeled pre-differentiated NPC could be detected, suggesting that pre-differentiation dramatically reduced the survival of seeded pre-differentiated NPC.

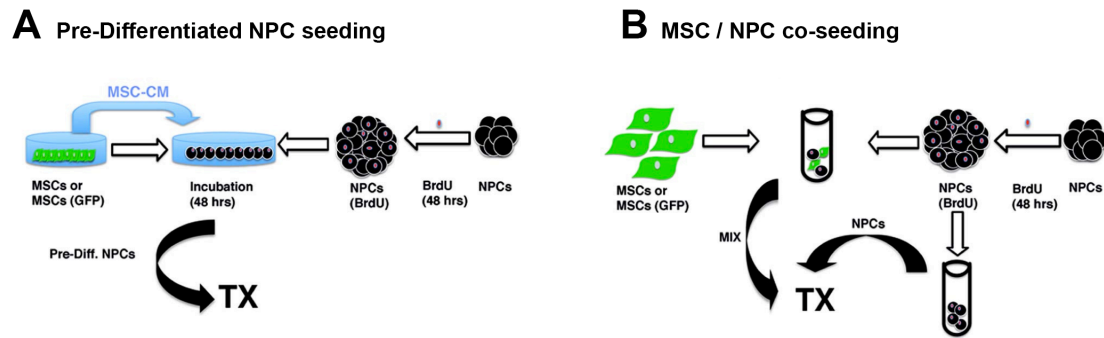


Figure 4.4. Schematic representation of the seeding paradigm

(A) Seeding of pre-differentiated NPC. NPC were labeled as neurospheres with BrdU under proliferative conditions for 48 hours. Then, these cells were plated on dishes and incubated with MSC-derived conditioned medium (MSC-CM) for 48 hours. After this period, BrdU-labeled NPC were seeded (TX) onto a hippocampal slice. (B) Co-seeding of MSC and NPC. BrdU-labeled NPC were mixed 1:1 with MSC or with eGFP-expressing MSC (MIX) and seeded onto a hippocampal slice. As a control, BrdU-labeled NPC were seeded alone (NPC). After 7 days, slices were analyzed for cell survival and differentiation.

4.6. Good survival of NPC after seeding onto hippocampal slices

In the next step, NPC were seeded together with MSC or alone onto hippocampal slice cultures. For this approach, the NPC were again pre-labeled with BrdU and mixed with the MSC immediately prior to the seeding procedure (Fig.4.4.B). After one week, slices were examined for the survival and differentiation of grafted NPC. Quantitative analysis of BrdU-labeled cells revealed that more than 40% of the seeded NPC survived either seeded alone or in combination with MSC (Fig.4.5.).

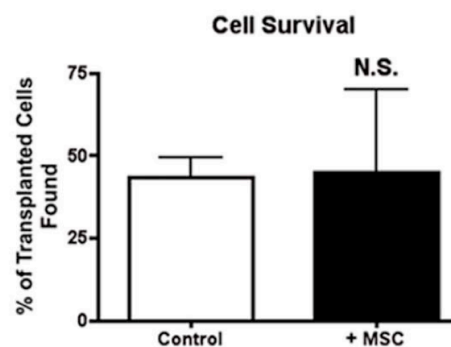


Figure 4.5. Survival of NPC and MSC seeded on hippocampal slice cultures

GFP-expressing MSC were co-seeded with NPC and incubated for 7 days. Quantitative analysis of survival of BrdU-labeled NPC expressed as the number of transplanted cells detected with respect to the total number of seeded cells. NPC were co-seeded with MSC (+ MSC) or alone (control). Data represent means \pm SD (standard deviation). Non-parametrical one-tailed Mann Whitney *t* test was used for statistical analysis (Rivera et al. 2009).

4.7. MSC promote oligodendroglial differentiation of co-seeded NPC on hippocampal slices

Cell fate determination of grafted NPC by confocal analysis indicated that most of the NPC co-seeded with MSC differentiated towards an oligodendroglial lineage, indicated by the oligodendroglia markers MBP and GST- π . In contrast, NPC seeded alone preferentially differentiated into GFAP positive astrocytes. In both groups, DCX expression could not be detected in the NPC grafts, indicating the absence of neuronal differentiation (Fig.4.6.).

Taken together, these data demonstrate that the pro-oligodendrogenic effect of MSC is maintained after co-seeding with NPC onto hippocampal slice cultures, confirming the previous *in vitro* results. Thus, co-transplantation of MSC with NPC might represent a promising strategy to replace lost oligodendroglia and to enhance remyelination after spinal cord injury.

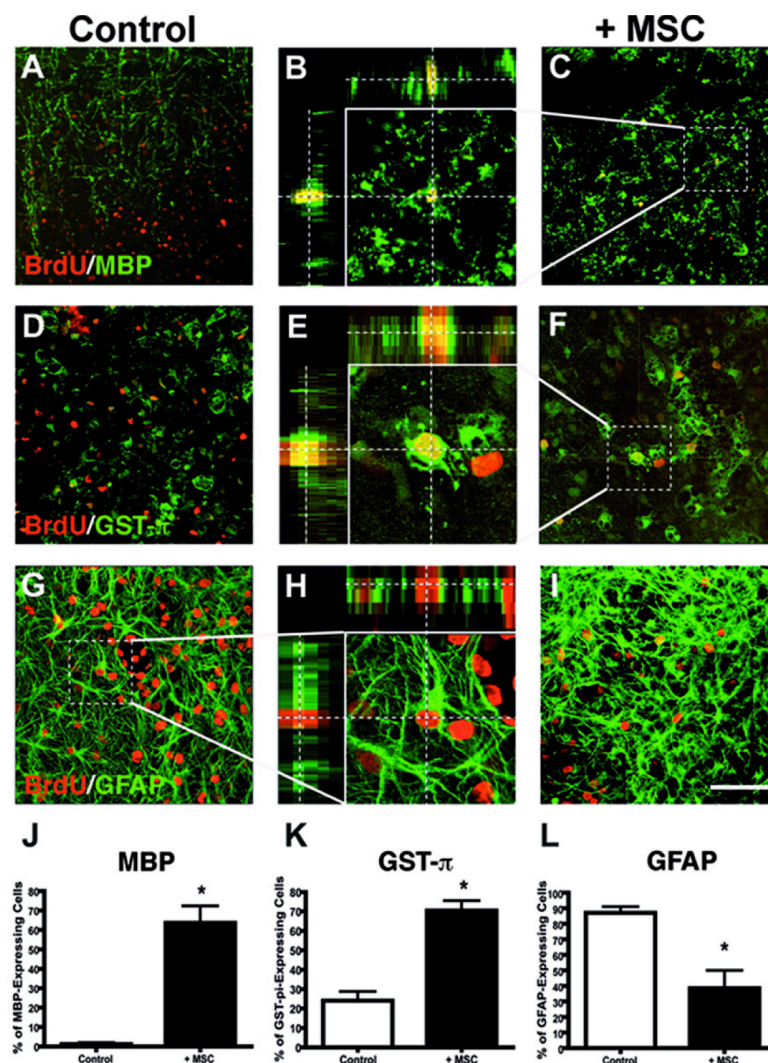


Figure 4.6. MSC promote oligodendroglial differentiation of NPC seeded on hippocampal slices

Seven days after cell seeding onto hippocampal slice cultures, the differentiation pattern of the grafted NPC was assessed, by co-localizing BrdU as a marker for seeded NPC (shown in red) with glial markers (shown in green). (A-I) Confocal images of grafted NPC alone (A, D, G, H) or together with MSC (B, C, E, F, I). NPC display immunoreactivity for MBP (green, A-C) and GST- π (green, D-F) for oligodendrocytes and GFAP (green, G-I) for astrocytes. Middle column (B, E, H) shows a higher magnification image including the Z-axis (C, F and G, respectively). Scale bar: 50 μ m. (J-L) Quantitative analysis of the expression of neural differentiation markers of the seeded NPC, graphs show the percentage of MBP- (J), GST- π - (K) and GFAP- (L) expressing cells. MSC enhance the percentage of MBP- and GST- π -expressing cells (oligodendrocytes) at the expense of GFAP-expressing astroglial cells. Experiments were done in triplicate. Data represent means \pm SD. Non-parametrical one-tailed Mann Whitney *t* test was used for statistical analysis; * *p* < 0.05 (Rivera et al. 2009).

4.8. Pre-differentiated NPC co-grafted with MSC promote oligodendroglial differentiation in the intact spinal cord

In the first set of experiments, NPC were grafted into the intact spinal cord. In order to detect the NPC after the transplantation, cells were pre-labeled with BrdU. NPC co-cultured with MSC for 3 days (**prediff-NPC/MS**) were compared with NPC and MSC mixed just prior to transplantation (see above; **NPC/MS**) into the intact dorsal column at cervical level C3. Pure NPC grafts (**NPC**) served as control. 10 days post-injection many BrdU-positive NPC were identified at and around the injection site in the dorsal column of the cervical spinal cord. Interestingly, NPC within NPC/MS grafts were much more confined to the injection site, whereas NPC as pure grafts migrated widely distributed along the rostro-caudal axis of the spinal cord (Fig.4.7.A-C). The absolute number of BrdU-prelabeled grafted NPC varied significantly within the different groups. NPC/MS grafts yielded 2768 ± 241.9 surviving NPC, whereas their number was reduced to 1407 ± 253 and 210 ± 37 NPC in the NPC and prediff-NPC/MS group, respectively (Fig.4.7.D).

The fate of grafted NPC was investigated by confocal analysis of the oligodendroglia marker APC and the astroglial marker GFAP. The percentage of BrdU-positive cells expressing APC (GFAP negative) or GFAP is shown as total of the three analyzed regions, ISWM, WM and GM. Indeed, in the prediff-NPC/MS group significantly more BrdU-positive cells expressed APC ($18.4\% \pm 2.4$) as a marker for oligodendroglial differentiation compared to the NPC and the NPC/MS group ($3.0\% \pm 1.5$ and $5.6\% \pm 1.6$, respectively).

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GFAP expression was reduced in the prediff-NPC/MSC group ($16.5\% \pm 0.8$) compared to NPC and the NPC/MSC grafts ($48.8\% \pm 3.0$ and $39.7\% \pm 4.2$, respectively) (Fig.4.7.E, F).

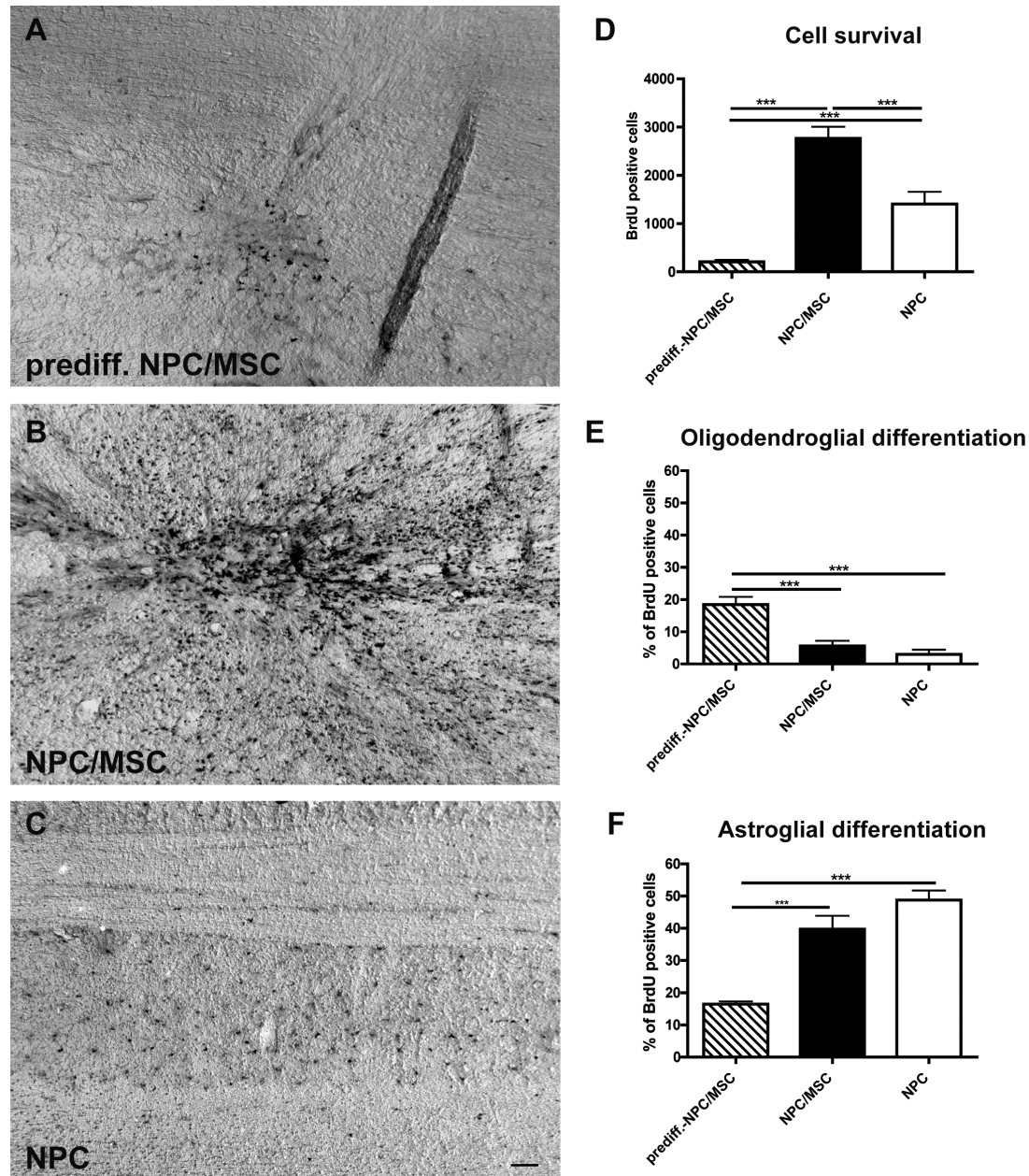


Figure 4.7. MSC promote an oligodendroglial fate of co-grafted pre-differentiated NPC in the intact spinal cord

NPC survival and differentiation was analyzed 10 days following transplantation into the intact spinal cord. (A-C) Brightfield images of sagittal BrdU-DAB stained sections; dorsal top; scale bar: 100µm. Immunodetection of BrdU prelabeled NPC reveals only few cells in prediff-NPC/MSC-grafts (A). Most of the BrdU-positive NPC were detected around the injection site in the dorsal column of the cervical spinal cord in NPC/MSC co-grafts (B). NPC were widely distributed along the rostro-caudal axis of the spinal cord in NPC only grafts (C). (D) Quantitative analysis of survival of BrdU-labeled NPC is expressed as the total number of

cells detected within the section containing the most cells. Significantly more NPC survived after co-grafting with MSC. (E, F) Quantitative analysis of the expression of neural differentiation markers of the grafted NPC was assessed by co-localizing BrdU as a marker for grafted NPC with glial markers GFAP (astrocytes) and APC (oligodendrocytes). Graphs show the percentage of APC- (E) and GFAP- (F) expressing cells. Averages are expressed with their standard error of the mean (SEM). Parametric one-way ANOVA was used for statistical analysis; *** $p < 0.001$.

4.9. MSC co-grafted with NPC fill the lesion site

The next experiments were done to investigate whether the pro-oligodendrogenic effect of the MSC seen *in vitro* is also present after co-transplantation of NPC with MSC into the injured spinal cord. Because the survival of pre-differentiated NPC was very limited after co-grafting with MSC into the intact spinal cord, NPC were not pre-differentiated for grafting into the injured spinal cord.

Previous studies have shown that fibroblasts sustain co-grafted NPC within the lesion center and allow complete tissue replacement (Vroemen et al. 2003, Pfeifer et al. 2004, Pfeifer et al. 2006). In the present study co-grafts of MSC and NPC (**NPC/MSC**) were grafted into the lesion site as mixed cell suspensions immediately after a C3 dorsal column transection. Animals receiving lesions only (**LESION**), pure NPC grafts (**NPC**), pure MSC grafts (**MSC**) or NPC/fibroblast co-grafts (**NPC/FF**) served as controls. Six weeks postoperatively, MSC containing grafts (**NPC/MSC** and **MSC**) replaced the cystic lesion defect identical to NPC/FF co-grafts, whereas NPC grafts failed to fill the spinal cord lesion defect, typically displaying a triangular or round cystic lesion cavity following a cervical wire knife dorsal column transection (Fig.4.8.A-E).

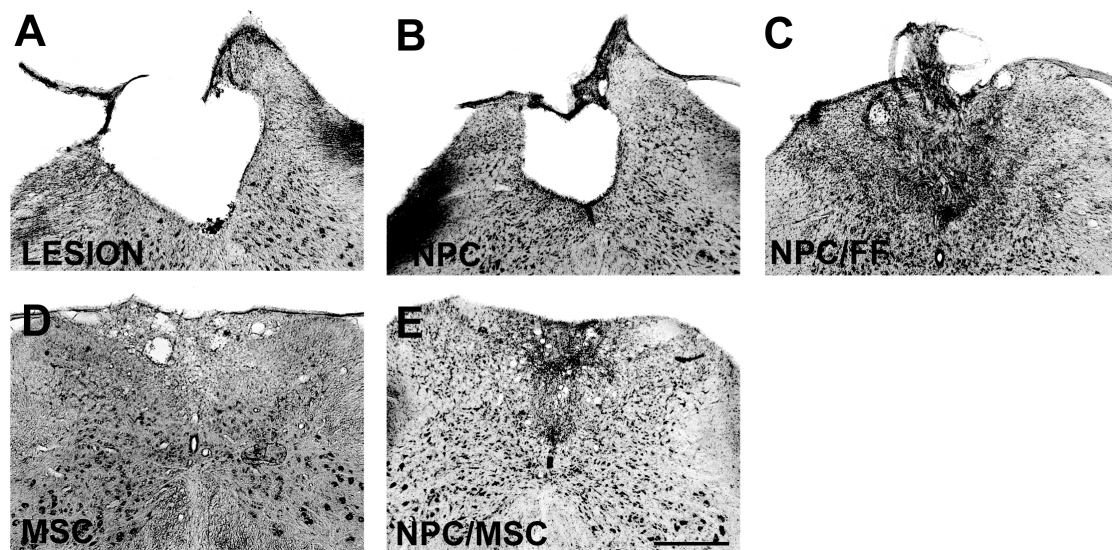


Figure 4.8. Cystic lesion replacement

(A) Six weeks following a cervical wire knife dorsal column transection a typical triangular shaped cystic lesion defect develops in animals with spinal cord lesion alone (B) or in combination with NPC grafts. (C) NPC/FF co-grafts, (D) MSC grafts as well as (E) NPC/MSC co-grafts replace the lesion defect completely. A-E: coronal Nissl stained sections, dorsal top; scale bar: 500µm.

4.10. MSC fail to promote oligodendroglial differentiation of co-grafted NPC in the injured spinal cord

The glial differentiation of NPC containing grafts (NPC, NPC/MSC, NPC/FF) was assessed six weeks postoperatively by co-localizing BrdU pre-labeled NPC with the astroglial marker GFAP and oligodendroglial marker APC (cells) in two different regions - dorsal column white matter (DCWM) and dorsal horn gray matter (GM) (Fig.3.1., Fig.4.9.A-H). Surprisingly, co-grafting with fibroblasts (NPC/FF), but not with MSC, significantly increased the number of grafted NPC expressing APC (GFAP negative) in the DCWM and GM (DCWM: NPC/FF: $27.1\% \pm 2.6$; NPC/MSC: $14.7\% \pm 1.9$; NPC: $13.6\% \pm 3.4$; GM: NPC/FF: $49.7\% \pm 13.4$; NPC/MSC: 13.9 ± 2.8 ; NPC: $12.7\% \pm 4.4$) (Fig.4.9.I,J). There was no statistically significant differences in the number of GFAP/BrdU double-labeled cells between all groups (Fig.4.9.I,J).

The differentiation of NPC at the lesion center can only be assessed in MSC or fibroblast containing co-grafts (NPC/MSC, NPC/FF), which replace the cystic lesion defect. We observed a trend towards enhanced oligodendroglial

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and astroglial differentiation within NPC/FF grafts compared to NPC/MSc grafts, which was not statistically significant (oligodendrocytes: NPC/FF: $33.5\% \pm 9.9$; NPC/MSc: $20.7\% \pm 3.0$; astrocytes: NPC/FF $60.3\% \pm 13.5$; NPC/MSc: $35.1\% \pm 9.8$) (Fig.4.9.K).

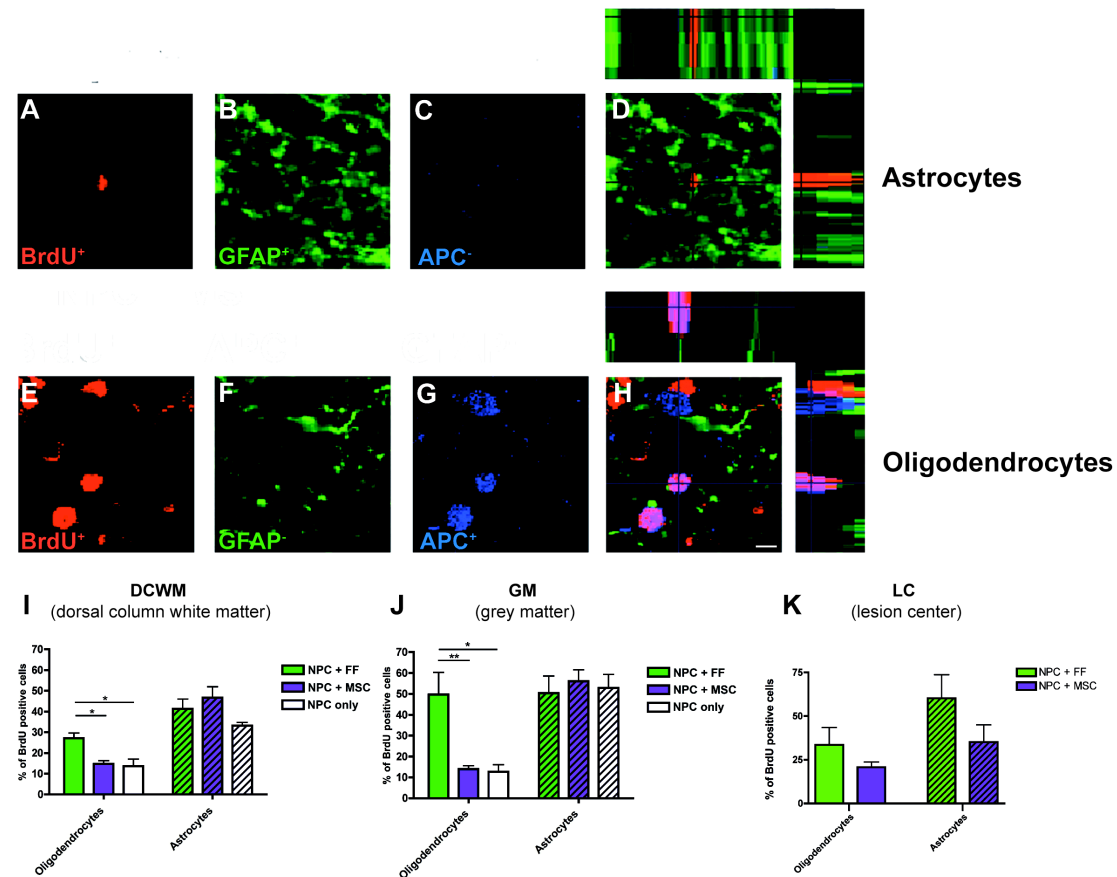


Figure 4.9. Analysis of cell differentiation in NPC co-grafted with MSC or fibroblasts into the injured spinal cord

(A-D) BrdU-prelabeled NPC co-localized with GFAP as an example of astroglial differentiation. (E-H) BrdU-positive NPC co-localized with APC (GFAP negative) as an example for oligodendroglial differentiation. (D, H) Respective merged micrographs; scale bar: 10µm. (I-K) Quantification of BrdU-prelabeled NPC co-localized with the glial markers GFAP or APC, in the dorsal column white matter (I), dorsal horn grey matter (J) and the lesion center (K). Fibroblasts enhance the percentage of APC-expressing cells in all regions analyzed. Averages are expressed with their standard error of the mean (SEM). Parametric one-way ANOVA was used for statistical analysis; * $p < 0.05$; ** $p < 0.01$.

4.11. Transplantation of MSC does not alter the proliferation of endogenous cells after spinal cord injury

Two different experiments were performed to investigate the proliferation and survival of newborn cells derived from endogenous NPC after spinal cord injury (Fig.4.10.). In order to examine the proliferation rate of endogenous NPC, animals received BrdU injections intraperitoneally once a day on 4 consecutive days, starting right after the animals received a cervical dorsal column transection followed by immediate cell transplantation of either GFP-positive MSC (**MSC**) or GFP-positive fibroblasts (**FF**). Three days postoperatively (**early time-point**), the amount of BrdU positive cells was assayed in 3 different sections (lesion center [LC], rostral [RO] and caudal [CA]) (for illustration see Fig.3.2).

Time course:

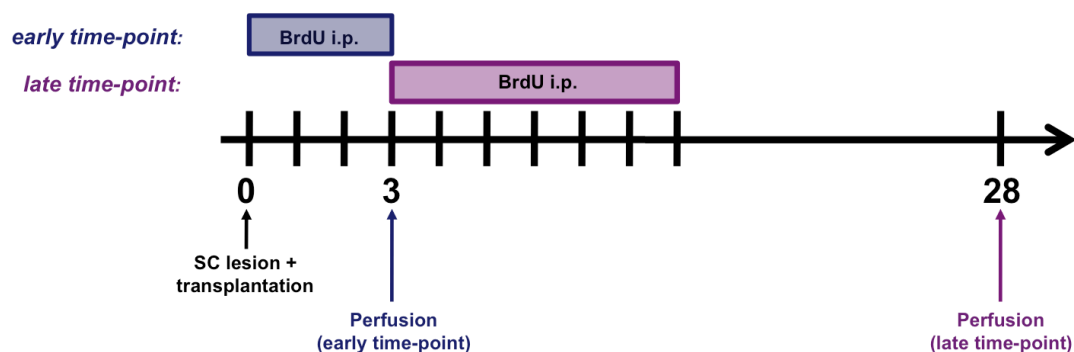


Figure 4.10. Schematic representation of the experimental design

All animals received a cervical wire knife dorsal column transection. Early time-point: Animals received BrdU injections intraperitoneally once a day on 4 consecutive days, starting right after the surgery. Perfusion of the rats was performed on post-op day 3. Late time-point: Rats received BrdU injections once a day for 8 consecutive days, starting on post-op day 3. Animals were perfused 4 weeks postoperatively.

Quantitative analysis of the BrdU-positive cells revealed that the transplantation of MSC seems to reduce the number of proliferating cells 3 days post-injury, even though the MSC did not alter the proliferation of the endogenous cells significantly compared to control animals. However, significantly more BrdU-labeled cells were found in the group receiving fibroblast grafts compared to the MSC group (Fig.4.11.A, C, E and G).

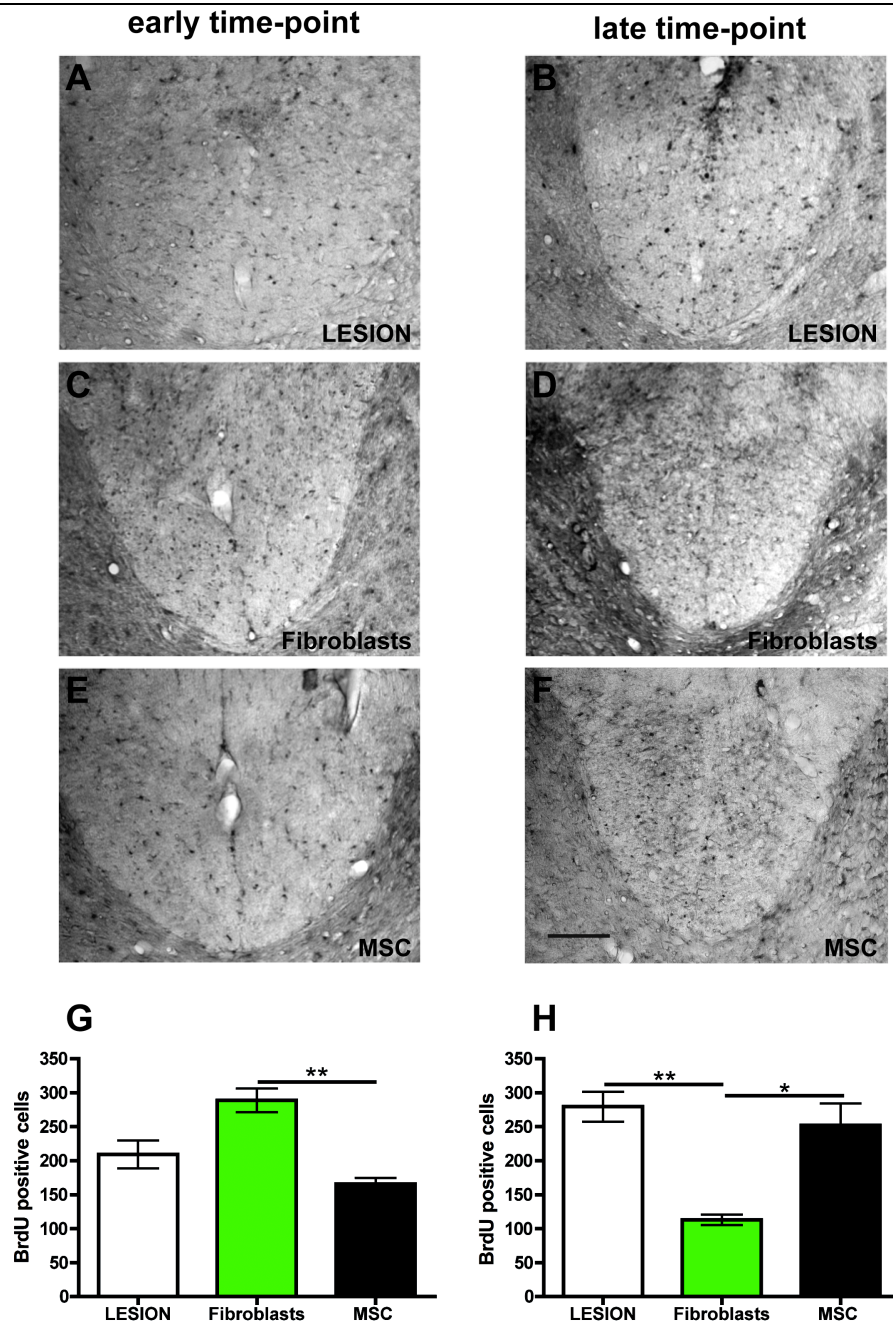


Figure 4.11. MSC do not alter the proliferation or survival of endogenous cells after spinal cord injury

Endogenous NPC proliferation and survival was analyzed 3 days, and 4 weeks postoperatively. (A-F) Brightfield images of coronal BrdU-DAB stained sections; dorsal top; scale bar: 100µm. Immunodetection of BrdU pre-labeled cells reveals more cells in the FF-group at the early time-point (B). At the late time-point, only few BrdU positive cells could be detected in the FF-group (E). Survival in the LESION (D) and MSC (F) group. (G, H) Quantitative analysis of BrdU-positive cells. The amount of BrdU positive cells is expressed as total of the three analyzed sections (lesion center [LC], rostral [RO] and caudal [CA]), whereas 4 regions each were analyzed in the rostral and caudal section. (G) The proliferation rate of the endogenous NPC was significantly reduced after grafting of MSC. (H) The survival of endogenous cells was significantly diminished after MSC-transplantation in comparison to the FF and LESION group. Averages are expressed with their standard error of the mean (SEM). Parametric one-way ANOVA was used for statistical analysis; * $p < 0.05$; ** $p < 0.01$.

In order to investigate the survival of the newborn endogenous NPC, animals received intraperitoneal BrdU injections once a day starting at day 3 until day 10 after the lesion/transplantation. The animals received MSC (**MSC**) or fibroblast (**FF**) grafts, whereas animals receiving only a lesion served as control (**LESION**). Four weeks postoperatively (**late time-point**), the number of BrdU positive cells was analyzed. Just like at the early time-point, the survival of the group which received MSC grafts was not altered compared to the control group. However, the survival of endogenous cells was significantly reduced in the FF-group compared to the two other groups. This effect was seen in all sections analyzed as well as in the total of the three sections. (Fig.4.11.B, D, F and H).

In summary these data suggest that transplantation of MSC does not alter the proliferation or survival of newborn cells directly after spinal cord injury compared to non-transplanted animals, while the transplantation of fibroblasts enhances the proliferation and decreases the survival of those cells.

4.12. MSC enhance endogenous oligodendroglial differentiation already within 3 days after SCI

Next, the glial differentiation of the newly generated endogenous NPC in the injured spinal cord was investigated with laser confocal microscopy. For this purpose, BrdU positive cells were co-localized with the glial markers APC (for oligodendroglia) and GFAP (for astroglia). The percentage of BrdU positive oligodendrocytes or astrocytes is shown as total of all analyzed regions/sections.

The expression of the oligodendroglial marker APC (GFAP negative) was already significantly increased three days postoperatively (**early time-point**) in the group receiving MSC-grafts (5.2 ± 0.9) compared to the FF-group (0.9 ± 0.2) or the control group (1.2 ± 0.2). Regarding the astroglial differentiation, a significant reduction of cells expressing GFAP in the MSC-group (51.6 ± 1.2) compared to the FF-group (59.9 ± 2.1) was found, whereas no alterations could be detected compared to the control group (56.7 ± 0.6) (Fig.4.11.A, B).

4.13. MSC grafts shift the differentiation pattern of endogenous NPC towards oligodendroglia four weeks after SCI

An enhanced oligodendroglial differentiation of the endogenous NPC could also be detected four weeks postoperatively (**late time-point**) in the group receiving MSC-grafts (7.1 ± 0.5) compared to the two other groups (FF: 0.8 ± 0.2 ; LESION: 1.5 ± 0.2). Moreover, significantly fewer cells expressed the astroglial GFAP in the MSC group (47.3 ± 1.0) compared to group receiving fibroblasts (58.1 ± 1.0) or to the control group (56.7 ± 1.1) (Fig.4.11.C, D).

These data indicate that MSC grafts enhance the oligodendroglial differentiation of endogenous NPC in expense of the astroglial differentiation after spinal cord injury (at the early and late time-point), recapitulating *in vitro* findings in cell culture (Rivera et al. 2006) as well as in hippocampal slice cultures (Rivera et al. 2009). However, fibroblast grafts failed to recapitulate the pro-oligodendrogenic effect observed *in vitro* and after co-transplantation with NPC into the injured spinal cord.

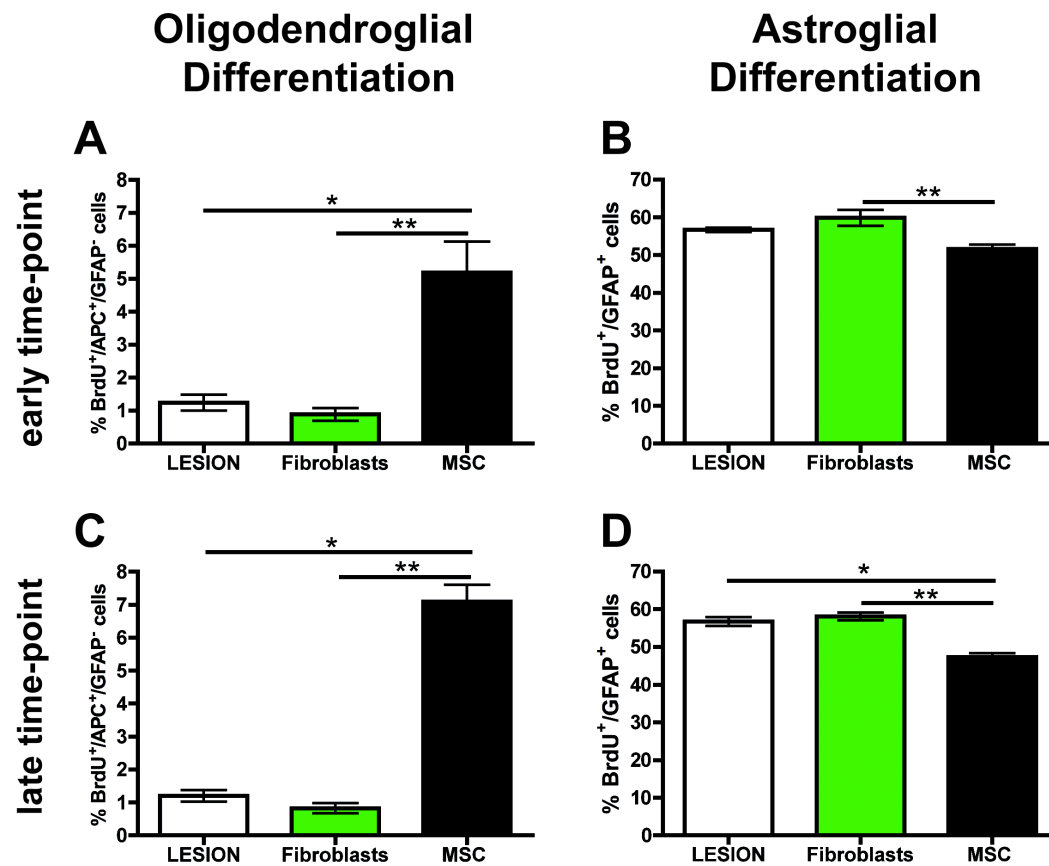


Figure 4.12. Oligodendrogenic effect of MSC on endogenous NPC

Quantitative analysis of the expression of neural differentiation markers of the endogenous NPC was assessed by co-localizing BrdU as a marker of newborn cells with the glial markers APC (oligodendrocytes) and GFAP (astrocytes), three days and four weeks after cell grafting. Graphs show the percentage of APC- (A, C) and GFAP- (B, D) expressing cells. MSC significantly enhance the percentage of APC-expressing cells (oligodendrocytes). Averages are expressed with their standard error of the mean (SEM). Parametric one-way ANOVA was used for statistical analysis; * $p < 0.05$; ** $p < 0.01$.

As shown above, MSC did not promote oligodendroglial differentiation in NPC after co-grafting into the injured spinal cord. We hypothesized that this could be caused by factor released in the acute spinal cord injury, which might counteract MSC derived soluble factors.

4.14. BMP2/4 block the effect of MSC-CM on cultured NPC

BMP2 and BMP4, which are highly up-regulated following spinal cord injury, are known as potent promoters of astroglial differentiation at the expense of oligodendroglial differentiation (Bonaguidi et al. 2005, Gross et al. 1996, Nakashima et al. 2001, Samanta and Kessler 2004). To test whether pro-oligodendrogenic effects of MSC-derived soluble factors could be inhibited by BMPs, NPC were cultured with MSC-CM alone or with MSC-CM + BMP-2/4. Additional controls were incubated with α -MEM-10% FBS + BMP-2/4 or α -MEM-10% FBS (Fig.4.13.A).

As suspected BMP 2/4 block the MSC-CM oligodendrogenic effect. The percentage of MBP expressing cells was drastically reduced when BMP2/4 were added to MSC-CM (MSC-CM+BMP 2/4: $9.0\% \pm 3.4$). Consistent with previous findings, MSC-CM alone promoted MBP expression in NPC cultures ($56.6\% \pm 9.5$; control conditions with α -MEM-10% FBS only: $6.1\% \pm 2.3$). Conversely, the percentage of GFAP-expressing astrocytes was significantly increased after adding BMP2/4 to MSC-CM treated NPC cultures compared to cells treated only with MSC-CM (MSC-CM + BMP 2/4: $83.8\% \pm 5.4$; MSC-CM: $20.8\% \pm 6.5$; control condition: $81.9\% \pm 2.4$). The quantification of Map2ab-expressing cells indicating neuronal differentiation was very low without significant differences between all conditions (data not shown).

To identify the molecular mechanism underlying the effects of BMPs to override MSC-derived oligodendrogenic effects, the expression of determinants known to participate in the oligodendrocyte/astrocyte fate decision of NPC was analyzed. The expression of the oligodendrogenic transcriptional factors Olig1 and Olig2 (Olig1/2) and the inhibitor of differentiation 2 (Id2), which has an anti-oligodendrogenic activity, was determined. It has been shown that Id2 can sequester Olig1/2 in the cytoplasm preventing their entry into the nucleus and DNA binding, therefore inhibiting the oligodendrogenesis and enhancing astrogenesis (Samanta and Kessler 2004) Moreover, the balance of Olig1/2 and Id2 determines oligodendrocyte/astrocyte fate decision.

NPC were incubated up to 7 days with MSC-CM in the presence or absence

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of BMP2/4. The expression level of Olig1, Olig2 and Id2 and Olig1/Id2 and Olig2/Id2 ratios were analyzed after 0, 3 and 7 days of incubation by quantitative RT-PCR. The Olig1/Id2 and Olig2/Id2 ratios were determined for the different conditions. Results showed that there is a significant change in both, Olig1/Id2 and the Olig2/Id2, ratios over time ($p < 0.001$, two-way-ANOVA). MSC-CM increased Olig1/Id2 and Olig2/Id2 ratios in NPC after 7 days ($p < 0.001$) of incubation. BMP 2/4 blocked this effect ($p < 0.001$) decreasing both ratios toward control condition levels (Fig.4.13.B+C). In summary, BMPs strongly inhibit the MSC derived oligodendrogenic activity by a mechanism that involves the balance of olig1/2 and Id2.

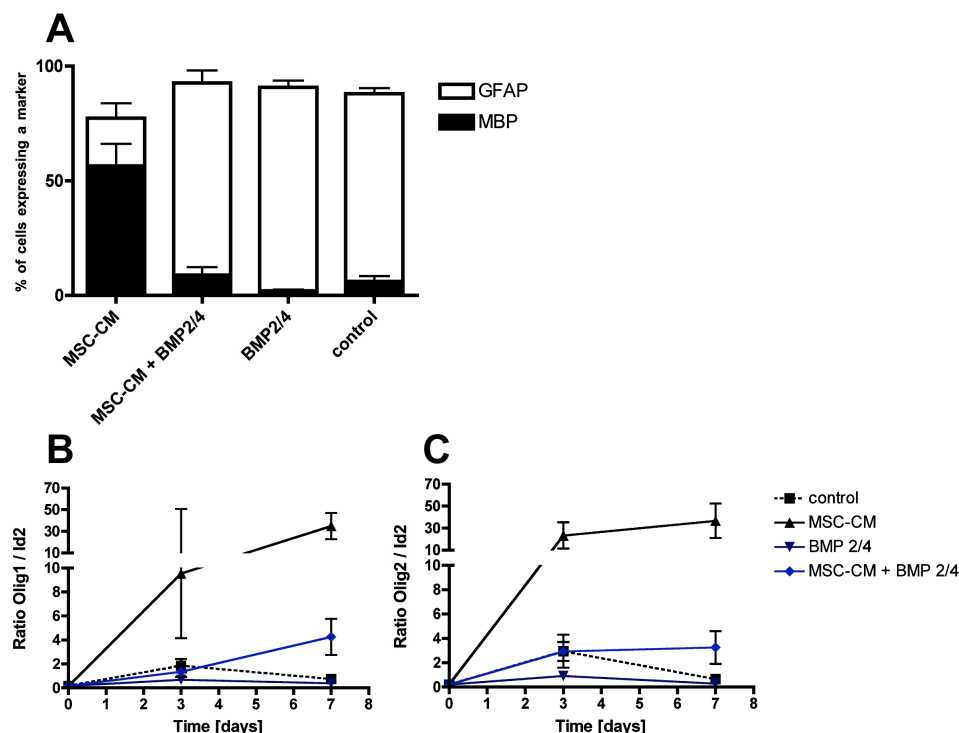


Figure 4.13. Anti-oligodendrogenic effect of BMP2/4 on cultured NPC

(A) NPC were incubated for 7 days in MSC-CM, MSC-CM+BMP2/4, α -MEM-10% FBS+BMP2/4 or in control medium (α -MEM-10% FBS). Quantitative analysis of the expression of neural differentiation markers (GFAP for astroglia; MBP for oligodendroglia). MSC-CM significantly enhanced the oligodendroglial differentiation, whereas the addition of BMP2/4 blocked this effect. Five hundred to 1,000 GFP-positive cells were analyzed in randomly chosen fields. Experiments were done in triplicate. Data represent standard error of the mean (SEM). Parametric one-way ANOVA was used for statistical analysis; *** $p < 0.001$. (B, C) NPC were incubated in MSC-CM, MSC-CM+BMP2/4, α -MEM-10% FBS+BMP2/4 or in control medium (α -MEM-10% FBS) for 0, 3 and 7 days. mRNA was prepared and quantitative RT-PCR was performed. Experiments were done in triplicate. Relative RNA concentrations with respect to a standard dilution curve versus time for Olig1, Olig2 and Id2 was calculated. Data express the Olig1/Id2 and Olig2/Id2 ratio over time. MSC-CM significantly enhanced the both ratios in NPC over time. Averages are expressed with their standard error of the mean (SEM). Two-way ANOVA was used for statistical analysis; *** $p < 0.001$.

4.15. Magnetic resonance (MR) imaging to analyze spinal cord injury in small animals non-invasively

Magnetic resonance imaging (MRI) represents the only noninvasive method to study the pathomorphologic changes in the injured human spinal cord parenchyma (Hadley and Teasdale 1988, Manelfe 1991). MRI is able to identify pathologic changes after spinal cord injury such as hemorrhage, edema, and secondary degenerative changes including cystic degeneration and fibroglial scar formation. In addition, MRI can be used to locate grafted cells, which have been labeled with special markers (e.g. iron oxide nanoparticles or gadolinium based probes). Various studies used dedicated experimental imaging systems with high-field strength to study morphological changes after spinal cord injury (Bilgen et al. 2000, Fraidakis et al. 1998, Ohta et al. 1999, Weber et al. 2006, Metz et al. 2000). However, the availability of high-field animal-dedicated systems is restricted. Unlike experimental imaging systems, clinical systems give access to a larger number of pulse sequences and allow directly comparing results between humans and rodents, since signal changes observed with experimental high-field MRI systems do not directly translate into findings obtained with clinical routine systems in humans. Overall, clinical systems are equipped with a more user-friendly interface, which helps to reduce setup time.

Therefore, the purpose of this study was to investigate the capacity of a broadly available routine clinical 3T MRI system equipped with a commercially available dedicated phased array spine surface coil to detect pathomorphological changes occurring in the adult rat spinal cord following contusion injury at the thoracic level or mechanical dorsal column transections at the cervical level.

4.16. MRI of the intact rat spinal cord

T1-weighted SE (spin-echo), T2-weighted TSE (turbo spin echo), and PD- (proton density) weighted TSE imaging with the 3T clinical MR scanner equipped with a dedicated four-channel rat spine phased array coil setup generated good-quality images of the uninjured spinal cord in vivo (Fig.4.14.) with an acquisition time between 6 and 10 minutes. The cerebrospinal fluid

appeared as a small hyperintense rim surrounding the spinal cord in T2-weighted TSE images (Fig.4.14.C). Only PD-weighted axial scans allowed a clear gray/white matter differentiation with the typical butterfly-shaped gray matter in axial scans (Fig.4.14.D).

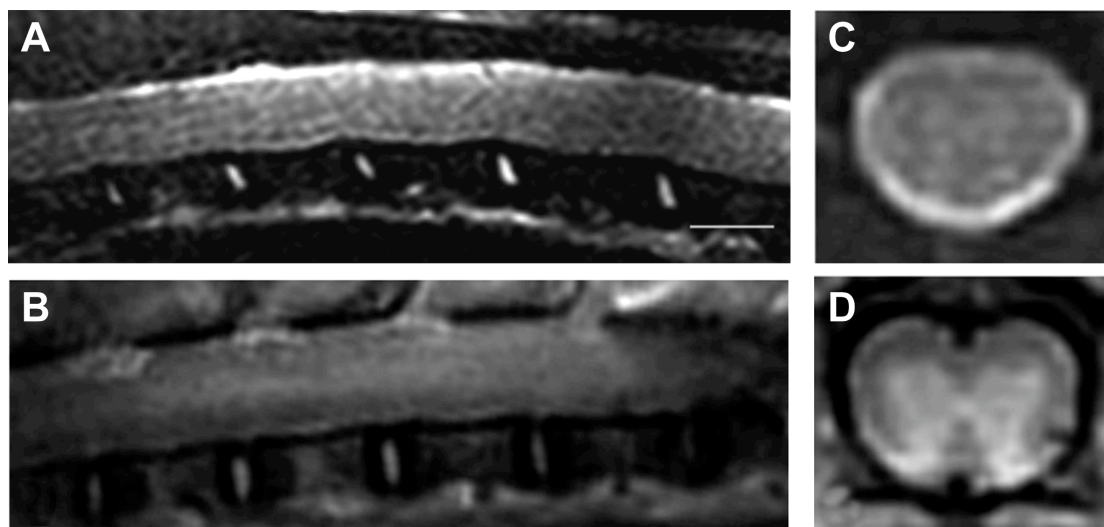


Figure 4.14. 3T MRI of the intact rat thoracic spinal cord in vivo

Shown is the intact thoracic rat spinal cord in T2- (A) and PD-weighted (B) sagittal scans. T2- (C) and PD-weighted (D) axial scans. In-plane resolution 200 x 200 μm . Scale bars: (A) 2.5 mm, (B) 3.2 mm, (C) 0.42 mm, and (D) 0.44 mm (Sandner et al. 2009).

4.17. MRI of the contused rat spinal cord

One day after spinal cord contusion injury, a total of eight injured rats underwent MRI. From these, four animals were perfused one day later. Another four animals were subjected to MRI again 43 days post injury. At day one post-injury, typical sequels of spinal cord trauma such as hemorrhage, tissue loss and beginning cyst formation in the center of the lesion could be detected consistently (Fig.4.15.A-C).

Most of the hemorrhage was confined to the gray matter. These findings were confirmed by correlating MR scans with corresponding Nissl stained sections (Fig. 4.15.D-F). At this early time point, hemorrhage-derived iron is not yet oxidized, which explains why the Prussian blue staining was consistently negative.

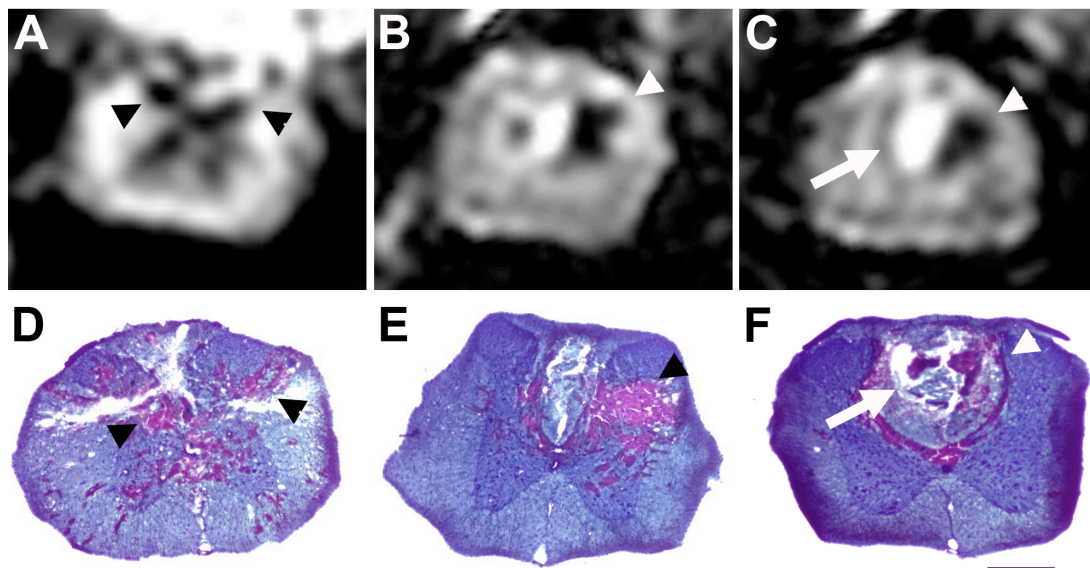


Figure 4.15. Axial images of the injured rat spinal cord in vivo one day post injury

(A-C) Axial T2 weighted MR images from the lesion center acquired on day one post injury. In-plane resolution 200 x 200 μm . (D-F) Corresponding Nissl stained coronal sections. Hypointense areas (arrowheads in A-C) in MR scans correspond to red appearing regions in Nissl stained sections (arrowheads in D, F) indicating trauma induced hemorrhage mostly confined to the spinal cord gray matter. In contrast, hyperintense signal changes (arrow in C) represent areas of cell debris, necrosis and cystic demarcation (arrow in F). Edema is represented by the faint hyperintense signal change surrounding the gray matter (A), which corresponds to the less intense Nissl-stained area of the spinal cord white matter (D) Scale bar (A-C) 0.86 mm, (D-F) 0.5 mm (Sandner et al. 2009).

At day 43 post injury, Prussian blue staining (Fig.4.16.B, 17C) specifically identified iron containing hemosiderin deposits, which could also be correlated with MR imaging, both in axial (Fig.4.16.A) and sagittal scans (Fig.4.17.A, B).

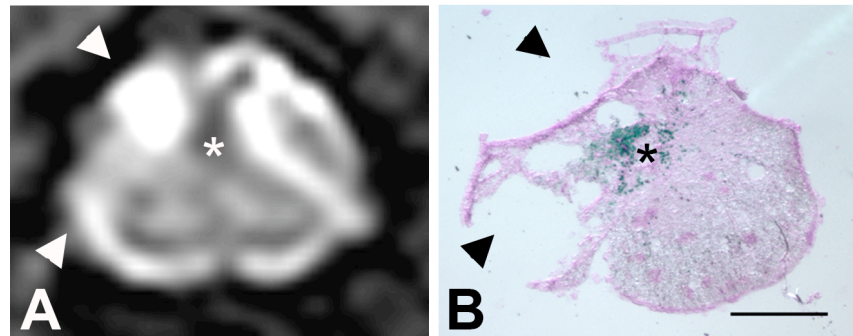


Figure 4.16. Axial images of the injured rat spinal cord in vivo 43 days post injury

(A) Axial T2 weighted MR image from the lesion center acquired on day 43 post injury. TSE, TR 2340 ms, TE 113 ms, section thickness 400 μ m, FOV 2.5 cm, in-plane resolution 200 x 200 μ m. (B) Corresponding Prussian blue stained coronal section. Areas with hyperintense signal changes (arrowheads in A) representing cystic degeneration (arrowheads in B) become more pronounced at the later time point investigated. The hypointense region (asterisk in a) corresponds to the Prussian blue positive location in the respective histological section (asterisk in B) indicating a previous hemorrhage. Scale bar (A) 0.78 mm, (B) 0.5 mm (Sandner et al. 2009).

Furthermore, due to the clearance of cell debris at the lesion site over time and consecutive cyst formation, areas with hyperintense signal changes were more pronounced and became more extensive corresponding to histological findings. At the late time point (day 43), spinal cord atrophy was detectable in axial and sagittal MR scans (Fig.16A, 20B).

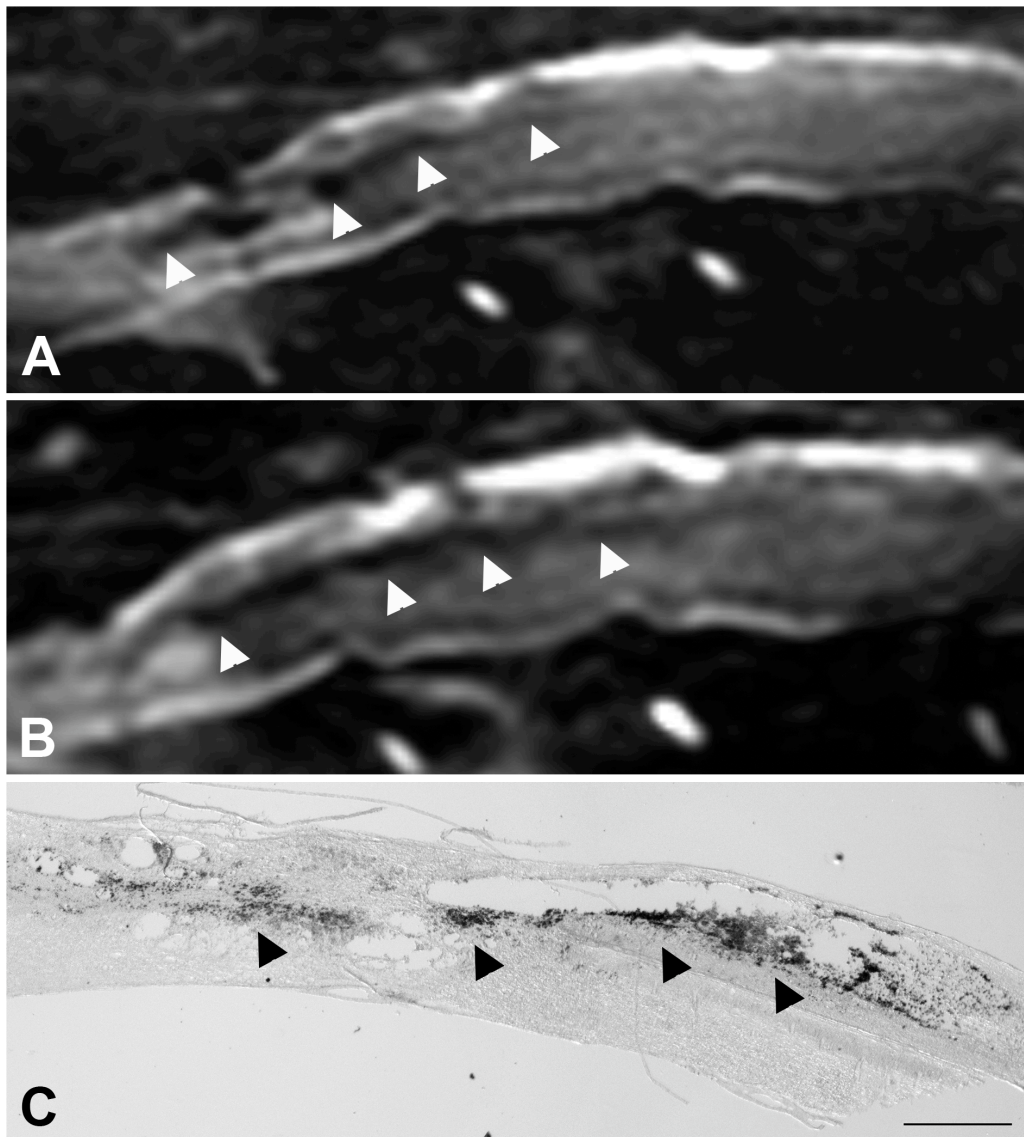


Figure 4.17. 3T MRI of the injured rat spinal cord in vivo 43 days post injury

(A, B) Sagittal T2 weighted MR images from the lesion center acquired on day 43 post injury from the same animal. TSE, TR 2340 ms, TE 113 ms, section thickness 400 μ m, FOV 2.5 cm, in-plane resolution 200 x 200 μ m. (C) Corresponding Prussian blue stained sagittal section. As seen in axial scans, hypointense signal changes representing hemorrhage (arrowheads in A, B) can be correlated with Prussian blue positive regions in sagittal sections (arrowheads in C). Regions of hyperintensity both within and around the spinal cord appear to enlarge over time, indicating the cystic degeneration at the injury site and the resulting spinal cord atrophy respectively. Scale bar (A) 1.77 mm, (B) 1.4 mm, (C) 1.0 mm (Sandner et al. 2009).

4.18. MRI of the rat spinal cord after cervical dorsal column transection

Representative T₂- and PD-TSE images in both coronal and sagittal planes of cervical lesion 30 days post-injury and their histology are provided (Fig.4.18.). T₂-TSE images clearly characterize cyst formation as confirmed by histological sections (Fig.4.18.A, C, E) whereas PD-TSE images portrayed injury along with a clear delineation of the gray-white matter structure confirmed by Nissl stained histology section (Fig.4.18.B, D, F).

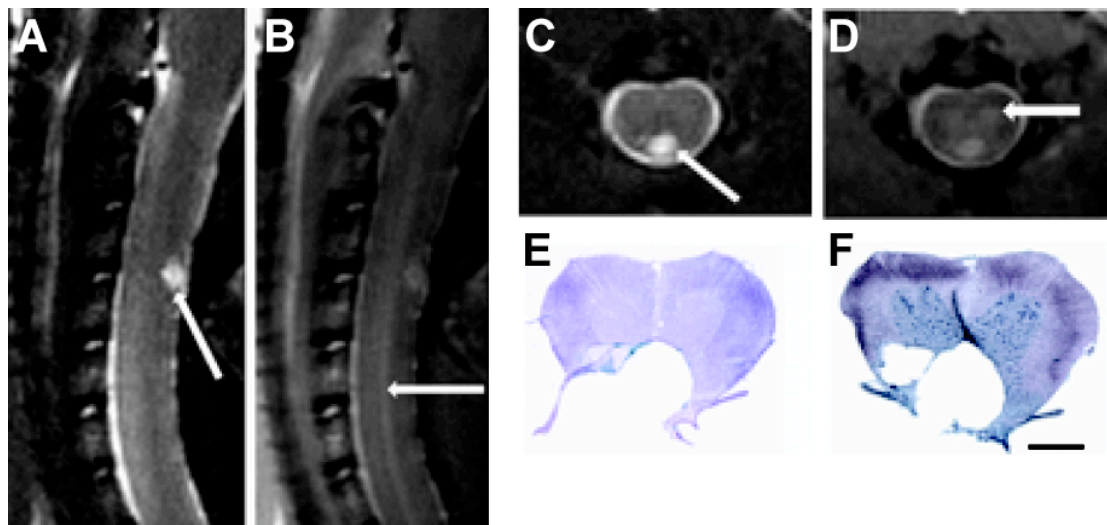


Figure 4.18. Tungsten wire knife induced cervical spinal lesion at 30-days post-injury

(A, C) Sagittal and axial T₂-TSE images. Arrows indicate fluid filled cyst as confirmed by (E, F) Prussian blue and Nissl stained histological sections. (B, D) Arrows highlight the detectable gray matter in sagittal and axial PD-TSE sections. Scale bar (C,D) 0.47; (E,F) 0.5mm.

5. Discussion

The results of the present study demonstrate, that mesenchymal stem cells (MSC) promote the differentiation and maturation of adult rat neural progenitor cells (NPC) towards an oligodendroglial fate (Rivera et al. 2006, Rivera et al. 2008). Co-transplantation of MSC together with NPC onto hippocampal slice cultures promoted oligodendrogenesis of the transplanted NPC. Moreover, co-transplantation of NPC, which have been pre-differentiated *in vitro*, together with MSC into the intact spinal cord also revealed an increased oligodendroglial differentiation of the grafted NPC. Similar results were obtained after transplantation of MSC into the injured spinal cord. MSC enhanced the differentiation of endogenous NPC towards an oligodendroglial fate at the expense of astroglial differentiation. The differentiation towards an oligodendroglial phenotype was not enhanced after NPC/MSC co-grafting into the injured spinal cord. As a likely underlying mechanism, BMP2 and BMP4, which are up-regulated after spinal cord injury, block the oligodendrogenic effect of MSC.

5.1. Determinants of graft differentiation in the injured spinal cord

Apparently an injury-induced differential expression of molecular factors yields different neural phenotypes. It is already known that the host environment rather than the intrinsic properties of the transplanted NPC determine the differentiation of grafted NPC (Shihabuddin et al. 2000, Gage et al. 1995a, Suhonen et al. 1996). For example, adult spinal cord derived NPC transplanted into the spinal cord differentiate exclusively into glial cells (Cao et al. 2002b, Cao et al. 2001, Enzmann et al. 2005, Pfeifer et al. 2004, Shihabuddin et al. 2000, Vroemen et al. 2003). In contrast, transplantation of the same type of NPC into a neurogenic region such as the hippocampus, induces predominantly neuronal differentiation (Shihabuddin et al. 2000). The question rises, which factor(s) might be responsible for the injury-associated determination of cell differentiation.

Potential candidates are bone-morphogenetic-proteins (BMPs) - in particular BMP2 and BMP4, which are strong promoters of astroglial differentiation at the expense of oligodendroglial and neuronal differentiation (Bonaguidi et al. 2005, Gross et al. 1996, Nakashima et al. 2001, Samanta and Kessler 2004). Moreover, BMPs are up-regulated in oligodendrocytes and astrocytes around the injury site (Matsuura et al. 2008) and have shown to promote glial scar formation (Setoguchi et al. 2004, Setoguchi et al. 2001, Fuller et al. 2007). Furthermore, *in vitro* experiments demonstrated that BMP2 and BMP4 inhibit the oligodendroglial and neuronal differentiation in cultured NPC and promote their astroglial differentiation (Bonaguidi et al. 2005, Gross et al. 1996, Nakashima et al. 2001, Samanta and Kessler 2004).

Strikingly, BMP2 and BMP4 block the MSC-CM derived effect. By adding BMP2/4 to the MSC conditioned media or to the control media, almost 90% of the cells differentiated into astrocytes and just about 2-8% obtained an oligodendroglial fate. Therefore, BMP2/4 are likely candidates to prevent MSC derived oligodendrogenic effect in the injury environment.

On the transcriptional level, BMPs are known to up-regulate the transcriptional regulator Id2, resulting in the inhibition of the pro-oligodendrogenic transcription factors Olig1 and Olig2 (Samanta and Kessler, 2004), which are required to induce oligodendroglial differentiation. Since Id2 and Id4 must reach a critical level in the cytoplasm to inhibit oligodendrogenesis through the binding of Olig1 and Olig2 to the DNA (Samanta and Kessler 2004), the Olig1/Id2- and Olig2/Id2-ratio was determined. A significant increase along time in the Olig1/Id2-ratio as well as in the Olig2/Id2-ratio in cells cultured in MSC-CM was detected. However, after incubation with BMP2/4 the Olig1/Id2- and the Olig2/Id2-ratio remained at the level of control conditions indicating that the BMPs block the pro-oligodendrogenic effect of MSC-CM. These findings strongly suggest that BMPs act also on the transcriptional level to neutralize MSC-CM induced effects.

This indicates, that the milieu at the injury site, where BMPs are up-regulated, might be at least in part, responsible for the lack of co-grafted NPC with MSC to differentiate into oligodendrocytes. It has been demonstrated, that the lesion site is mainly infiltrated by fibronectin-positive cells, whereas GFAP-positive cells up-regulate among others BMP4, in the tissue adjacent to the

lesion center (Chen, Leong and Schachner 2005). This is in accordance with preliminary data from our group, also indicating that BMP2/4 are predominately up-regulated in the tissue adjacent to the graft/lesion center. NPC, which have been grafted directly into the lesion site, are close to cells expressing BMPs. Hence, the effect of BMPs might be stronger than the effect of the grafted MSC, therefore inhibiting the oligodendroglial differentiation of the grafted NPC, just as my *in vitro* data indicate.

The fact that grafted MSC still have a strong pro-oligodendrogenic effect on endogenous NPC, might be due to the location of the endogenous NPC. Endogenous NPC are not located in the lesion site like the grafted NPC, but rather get attracted from the adjacent host parenchyma. Therefore, it might be possible that these cells do not get affected as strong as the grafted NPC by the locally increased BMPs. Since MSC release a soluble factor, it might be possible that this factor is still be able to reach the endogenous NPC, inducing an oligodendroglial differentiation.

MSC could not induce the oligodendroglial differentiation of co-grafted NPC into the intact spinal cord most likely due to the fact, that even though the animals did not underwent a spinal cord transection, the cell injection itself causes some inflammation and the up-regulation of BMPs. Therefore, the presence of BMPs in close relation to the grafted cells might again inhibit the pro-oligodendrogenic effect of the MSC. Since pre-differentiated NPC showed an enhanced oligodendroglial differentiation, this further indicates that once the NPC are committed towards an oligodendroglial fate, they are not affected by BMPs anymore. However, further studies need to be done to confirm these explanations.

Both, MSC and FF grafts filled the cystic defect developing after SCI and provided a supporting scaffold to maintain adult NPC within the lesion cavity in line with previous studies (Lu et al. 2005, Pfeifer et al. 2004, Vroemen et al. 2007). Surprisingly FF, introduced as a control condition, significantly increased the expression of oligodendroglial marker (>30% of co-grafted NPC) in co-grafted NPC. In a previous study employing NPC/FF co-grafts only 18% of the co-grafted NPC differentiated into oligodendrocytes (Pfeifer et al. 2004). There, post-transplantation survival time was only 3 weeks compared to 6 weeks in the present study, which might explain the

differences. It is conceivable, since both, FF and MSC belong to the mesenchymal lineage (Haniffa et al. 2008, Cappellesso-Fleury et al. 2010) that the same factor is responsible for promoting oligodendroglial differentiation. Fibroblasts may express this yet unknown factor at higher level or express additional factors, which facilitate oligodendroglial differentiation in addition. So far, a number of growth factors, cytokines, and hormones have been excluded as potential oligodendrogenic factors in the MSC-CM: insulin-like growth factor-1 (IGF-1), thyroid hormone (TH), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), nerve growth factor (NGF), transforming growth factor β -1 (TGF β 1), neurotrophin-3 (NT-3), Shh, PDGF-AA, UDP-glucose, and Noggin (Rivera et al. 2006, Rivera et al. 2008).

My *in vitro* experiments demonstrated that the pro-oligodendrogenic effect of MSC-CM on NPC was even more pronounced in FF-CM. The combination of MSC-CM with FF-CM did not lead to an additive or over-additive effect in terms of astroglial or oligodendroglial differentiation, therefore it might be possible that factors secreted by MSC or fibroblasts stimulate at least in part the same signaling pathway. Another possibility might be that either more than one FF-CM derived factor is inducing this pro-oligodendrogenic/anti-astrogenic effect or fibroblasts express the yet unknown factor in higher concentrations.

Another study focusing on wound healing demonstrated that paracrine factors released by MSC and fibroblasts or by their conditioned medium revealed different expression levels in response to injury. The factors released by MSC have shown to recruit significantly more macrophages and endothelial lineage cells into the wound, thereby enhancing wound healing, compared to fibroblast-derived factors (Chen et al. 2008). These results demonstrate, that even though MSC and fibroblasts release the same factors, it might be that these factors act differently on the differentiation of NPC.

The analysis of endogenous NPC differentiation after SCI revealed that the fibroblast grafts did not enhance the differentiation of endogenous NPC towards oligodendrocytes. However, MSC grafts enhanced the number of APC-expressing oligodendroglia. Although APC-positive oligodendrocytes have been shown to represent a mature phenotype (Bhat et al. 1996, Fuss et al. 2000, Tripathi and McTigue 2007) (Fig.5.1.), which can promote remyelination (Cai et al. 2010, Mela and Goldman 2009, Cao et al. 2010, Chambers and Perrone-Bizzozero 2004), it needs to be assessed whether APC-positive oligodendrocytes remyelinate axons in the described experiments.

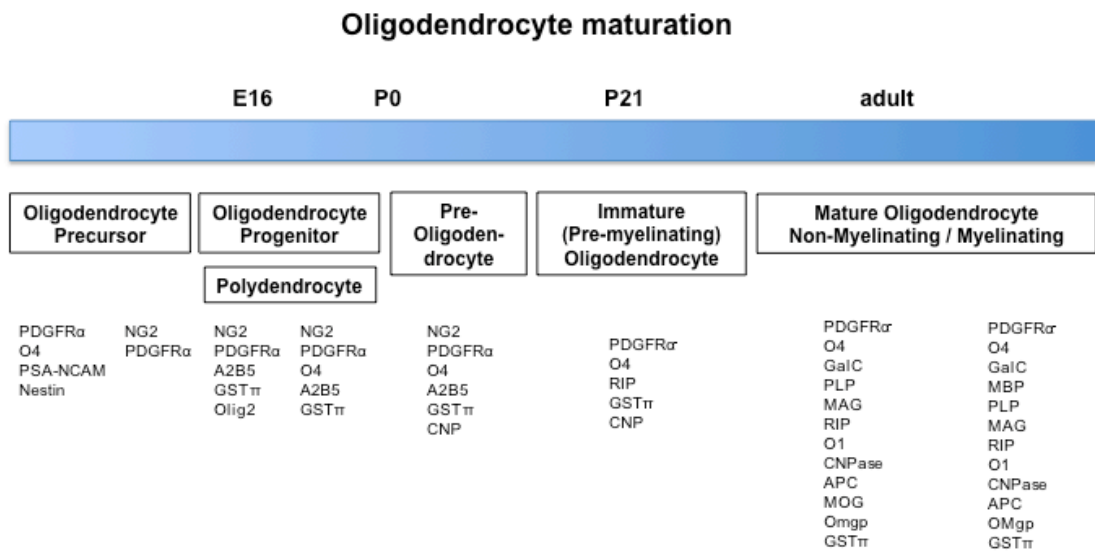


Figure 5.1. Oligodendrocyte maturation markers

During development, a progressive differentiation from oligodendrocyte precursor to mature oligodendrocytes is observed. Stage specific expression of markers are listed. (Figure adopted from (McDonald and Belegu 2006, de Castro and Bribian 2005, Nishiyama 2007)

The fact, that fibroblasts did not promote oligodendroglial differentiation in endogenous NPC might be due to the fact that endogenous NPC do not respond in the same way to soluble factor(s) derived from fibroblasts than transplanted NPC.

In animals receiving fibroblast grafts cell proliferation was increased 3 days postoperatively compared to MSC grafted animals. However, the number of surviving newborn cells (determined by quantification of BrdU at 4 weeks survival time-points) was significantly reduced in FF grafted spinal cords

compared to MSC grafted animals. Syngenic FF grafts could induce a strong inflammatory response early on (reflected by an increased number of proliferating cells). Over time, the early inflammatory response may create an unfavorable milieu for newborn cells. As a consequence, an increased number of endogenous NPC undergoes cell death. The situation is different in MSC grafts. Known immunomodulatory effects of MSC (Caplan 2007, Uccelli et al. 2008, Uccelli et al. 2007, Uccelli et al. 2006) diminish the recruitment of inflammatory cells in the beginning (3 day survival time-point), resulting in less proliferating cells, which leads in the long term to better survival of endogenous NPC indistinguishable from animals with lesions only. Further studies need to confirm these explanations.

5.2. Graft survival excludes proper cell differentiation and vice versa

Previous studies demonstrated that the stimulation of NPC with MSC-CM for 2-3 days sufficiently induces oligodendrogenic commitment in NPC (Rivera et al. 2006, Rivera et al. 2008). Therefore, NPC were incubated with MSC-CM for 3 days prior to transplantation into the intact spinal cord. Indeed, significantly more pre-differentiated NPC expressed oligodendroglial marker after transplantation into the intact spinal cord compared to NPC, which were not pre-differentiated. Apparently the pre-differentiation of NPC is mandatory to maintain oligodendroglial differentiation after transplantation, however, at the expense of NPC graft survival. These results – favorable cell survival prohibits the desired cell differentiation and vice versa - once again illustrate the dilemma of stem/progenitor cell transplantation. This is in agreement with previous studies, in which cells transplanted at early stages of the oligodendroglial lineage, like oligodendrocyte progenitors, showed a better survival and a greater capacity for migration in rodent models of focal demyelination, compared to cells, which are already more differentiated towards the oligodendroglial lineage (Archer et al. 1997, Warrington, Barbarese and Pfeiffer 1993, Windrem et al. 2004). The same applies for human neural precursor cells, which have been pre-differentiated before transplantation into the intact adult rat hippocampus – the undifferentiated cells showed a better survival than the pre-differentiated ones (Le Belle,

Caldwell and Svendsen 2004). One solution to overcome this problem could be NPC grafts, which have been genetically modified to introduce regulatable transgenes in a temporally controlled manner (e.g. with tetracycline-regulated expression systems), which express pro-oligodendrogenic transcription factors such as Olig1 and Olig2. Undifferentiated NPC are allowed to survive and integrate into the host parenchyma before Olig1/2 expression is turned on to promote oligodendroglial differentiation.

Previously, adult NPC have been genetically modified to express neurogenin-2 (Ngn2) before transplantation into the injured spinal cord (Hofstetter et al. 2005). Ngn2 is a pro-neural transcription factor that promotes neuronal and inhibits astroglial differentiation (Nieto et al. 2001). However, Ngn2 expressing NPC grafts showed enhanced oligodendroglial differentiation compared to naive NPC. In this case, pre-differentiation did not negatively influence graft survival. Grafted Ngn2 expressing NPC adopted a more immature oligodendroglial phenotype, which might explain the favorable cell survival. Over time, grafted Ngn2 expressing NPC prevented dysmyelination and increased remyelination (Hofstetter et al. 2005).

5.3. Responsivity of different neuroanatomical regions to pro-oligodendrogenic cues

At first, an increased oligodendroglial differentiation was observed after adding MSC-CM to rat hippocampus-derived NPC (Rivera et al. 2006). Here it is shown that rat NPC from different regions of the CNS are equally responsive to MSC-CM to differentiate into oligodendroglia. Both, SVZ and spinal cord derived NPC strongly increase oligodendroglial marker expression after co-cultivation with MSC or incubation with MSC-CM. This is supported by recent findings showing that adult rat SVZ derived NPC do not differ from spinal cord derived NPC in terms of the percentage of neuronal, astroglial and oligodendroglial differentiation (Kulbatski and Tator 2009). Considering autologous NPC transplantation as a potential clinical application this is good news. The SVZ and the spinal cord represent a much more accessible region to obtain tissue for NPC isolation compared to the hippocampus.

Several studies described an increased proliferation of endogenous neural progenitor cells following spinal cord injury (Yamamoto et al. 2001, Xu et al. 2006, Zai and Wrathall 2005, Horky et al. 2006, McTigue et al. 2001). Therefore, the recruitment and modulation of endogenous NPC towards an oligodendroglial fate as shown here with the transplantation of MSC might represent an alternative strategy in order to promote functional outcome following spinal cord injury.

Here enhanced oligodendroglial differentiation with MSC and FF grafting has been described. However, this does not yet say anything about remyelination and restoration of nerve conduction with this approach. Several studies emphasized that the presence of endogenous oligodendrocyte precursor cells within the injured or demyelinated spinal cord does not automatically mean remyelination (Karimi-Abdolrezaee et al. 2006, Sher et al. 2008). A clear demonstration that the generated cells are able to differentiate into mature, myelin-producing oligodendrocytes is missing (Levine and Reynolds 1999). In the present study the analysis of toluidine blue stained semi-thin sections did not provide evidence of enhanced remyelination after MSC grafting. However, the dorsal column transection model is most likely not an appropriate model to study demyelination following spinal cord injury. Studies comparing spinal cord contusion and transection demonstrated that the extent of myelin pathology differs following contusion and transection injury. A hemisection injury resulted in focal only demyelination, whereas a contusion injury resulted in a more widespread demyelination at greater distances from the lesion center (Siegenthaler, Tu and Keirstead 2007). Therefore, the next step is to investigate remyelination in a contusion model. A contusion injury leads in addition to the mechanical damage to the spinal cord also to displacement of tissue at a distance from the site of injury, which results in more severe and longer lasting locomotor deficits than in a transection model. Hence, contusion injuries reproduce more closely anatomical and behavioral outcomes occurring in patients suffering from spinal cord injury (Basso et al. 1996, Hamers et al. 2001, Siegenthaler et al. 2007).

5.4. Feasibility of a clinical 3T MRI scanner to study pathological changes occurring after spinal cord injury in the rat

Results from the MRI study demonstrate that a clinical 3T MR imaging system with a dedicated phased array spine surface coil allows the visualization of neuropathological sequels of spinal cord contusion injury in the living rat. So far, this is the first time that a 3.0T MR scanner designed for routine clinical studies has been employed to obtain high quality images from the rat spinal cord.

The employed clinical 3T imaging system achieves a spatial resolution that is roughly 8 times lower compared to the 17.6T MRI (Weber et al. 2006). Until now, the 17.6T MRI system yielded the best resolution for MRI of the rat spinal cord. However, compared to experimental imaging systems that operate in a similar range of field strength (1.5– 4.7T), the spatial resolution of the clinical 3T scanner is almost equivalent to systems with a field strength up to 4.7T (Metz et al. 2000, Ohta et al. 1999, Fukuoka et al. 1998b, Guizar-Sahagun et al. 1994, Fraidakis et al. 1998). Even compared to previous studies using implantable coils (Narayana et al. 2004, Bilgen et al. 2000, Ford et al. 1994) requiring additional invasive surgery the difference in spatial resolution is rather small.

The spatial resolution in PD-weighted images is very important in the spinal cord as it is the only one sequence that can clearly delineate the gray and white matter tracts of the intact rat spinal cord in vivo. The loss of gray-white matter contrast is the hallmark of spinal contusion injuries, which is invariably associated with lower body paralysis. The return of gray-white matter contrast in images acquired by PD-TSE sequences therefore serves as a surrogate marker for clinical improvement (Narayana et al. 2004). With T2-weighted images, which are known to detect hemorrhage and edema in the acute stage as well as cystic degeneration in the subacute/chronic stage (Flanders et al. 1990, Hackney et al. 1986, Kulkarni et al. 1987), we could clearly identify these sequels of spinal cord injury with 3T MRI. The MRI findings were confirmed by analysis of corresponding histological sections. Of course, the spatial allocation of respective signal changes is not as precise as observed

with 17.6T MRI; however, it is sufficient to serve as a morphological outcome parameter or to help find the optimal transplantation site in the context of cell- or biomaterial-based spinal cord repair strategies (Pfeifer et al. 2004, Prang et al. 2006).

In general, high field strength MRI systems are superior to the 3T clinical scanner in terms of spatial resolution. Pathomorphological changes following experimental spinal cord injury in small animals can be detected more precisely. However, not all systems are suitable for imaging of the adult rat spinal cord in vivo. For example, with 17.6T MRI the mortality rate was high due to the small bore size and insufficient air/anesthetic circulation, which makes studies over time with identical animals not really feasible. In the present study with the 3T MR scanner, there was not a single animal lost resulting from the imaging procedure. With this system, rats weighing up to 400 g can be analyzed. Of course, high-field animal-dedicated scanners in the range from 4.7 up to 11.7T with larger bore diameters can also be applied for adult rats without major restrictions in animal size. Unfortunately, these experimental high-field imaging systems are only available in dedicated imaging centers.

In conclusion, the employed routine clinical 3T MR scanner in combination with a dedicated phased array spine surface coil allows the noninvasive visualization of morphological changes occurring in the injured rat spinal cord in vivo at a spatial resolution that thus far could only be achieved with experimental MRI systems at higher field strength.

5.5. Summary and Conclusion

As shown here, MSC represent promising cellular candidates to promote oligodendroglial differentiation in endogenous glial progenitor cells proliferating after spinal cord injury. The functional relevance in terms of remyelination and restoration of nerve conduction has yet to be determined in a relevant spinal cord injury model. Further efforts need to identify the soluble factor(s) responsible for the observed oligodendrogenic effects. Besides, the creation of cellular bridges and reduction of the glial scar through MSC, by having anti-inflammatory effects, e.g. through inhibiting the proliferation of T

and B lymphocytes (Uccelli et al. 2007), MSC have some axon-regenerative aspects (Cho et al. 2009) and show positive effects on the vasculature (Parr, Tator and Keating 2007), which would be important for a possible clinical application. Moreover, MSC can be easily genetically modified to over-express certain growth factors and are therefore suitable cell types for ex vivo gene delivery (Lu et al. 2005, Lu et al. 2004). Others have demonstrated that MSC grafts establish a cellular matrix that supports host axonal growth into but not beyond spinal cord lesion sites (Lu et al. 2005, Lu, Jones and Tuszynski 2007, Ankeny et al. 2004, Hofstetter et al. 2002, Neuhuber et al. 2005). Therefore, combinatorial approaches will be necessary to achieve axonal growth beyond the lesion site and functional recovery (Lu and Tuszynski 2008, Lu et al. 2004, Pearse et al. 2004). Taylor et al. demonstrated that grafts of MSC in combination with a gradient of NT-3 rostral to the lesion site allows sensory axons to bridge for short distances beyond the lesion site (Taylor et al. 2006). Subsequent studies demonstrated that the distance of sensory axon regeneration is increased when a gradient of NT-3 is combined with the stimulation of regenerative responses at the cell soma (Alto et al. 2009). Other approaches demonstrated that the combined stimulation of neural cells bodies with cAMP and the stimulation of injured axons with NT-3 promotes axonal regeneration (Lu et al. 2004). Taken together, these unique properties of MSC make them a favorable cell type to be developed further towards clinical translation.

Finally, a clinical 3T MRI scanner was successfully employed to non-invasively visualize morphological changes occurring after spinal cord injury in the rat, which is a prerequisite for translating cell-based therapies into the clinic.

6. List of Abbreviations

α -MEM	Alpha Modified Eagle Media
ANOVA	Analysis of Variance
APC	Adenomatous polyposis coli
ASIA	American Spinal Injury Association
AT	Ascending Tract
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
BMP 2/4	Bone Morphogenetic Protein 2/4
BrdU	5-Bromo-2'-Deoxyuridine
BSA	Bovine Serum Albumin
CA	Caudal
GalC	Galactocerebroside
CAP	Cortical Cytoskeleton-associated Protein
CC	Central Canal
cDNA	Complementary DNA
ChABC	Chondroitinase ABC
CNPase	2',3'-Cyclic Nucleotide 3'-Phosphodiesterase
CNS	Central Nervous System
CPS	Cryo Protect Solution
CSF	Cerebrospinal Fluid
CSPG	Chondroitin Sulfate Proteoglycan
CST	Corticospinal Tract
Cy5	Cyanine 5
DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-phenylindole
DCWM	Dorsal Column White Matter
Dk	Donkey
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl Sulfoxide

List of Abbreviations

DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
DREZ	Dorsal Root Entry Zone
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
eGFP	enhanced Green Fluorescent Protein
EPHB2	Ephrin-B2
EPO	Erythropoietin
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FF	Fibroblast
FF-CM	Fibroblast-Conditioned Media
FOV	Field Of View
FSBG	Fish Skin Gelatin Buffer
G6PDH	Glucose-6-Phosphat-Dehydrogenase
Galc	Galactosylceramidas
GAG	Glycosaminoglycan
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GM	Grey Matter
GM/WM	Grey matter / White Matter transition zone
GPI	Glycosylphosphatidyl
GAP	Growth Associated Protein- π
GST- π	Glutathione S-transferase
GTP	Guanosintriphosphat
HC	Hippocampus
HIV	Human immunodeficiency virus
Id2/4	DNA-binding protein inhibitor 2/4
IgG	Immunoglobulin, isoform G
iNOS	inducible Nitric Oxide Synthase
i.p.	intraperitoneally
ISWM	Injection Site in the White Matter

List of Abbreviations

LC	Lesion Center
LCWM	Lateral Column White Matter
Map2ab	Microtubule-associated Protein 2 Isoform a+b
MAG	Myelin-Associated Glycoprotein
MBP	Myelin Basic Protein
MOG	Myelin Oligodendrocyte Glycoprotein
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
Ms	Mouse
MSC	Mesenchymal Stem Cell
MSC-CM	Mesenchymal Stem Cell-Conditioned Media
NB	Neuro Basal
NCAM	Neural Cell Adhesion Molecule
NG2	Chondroitin Sulfate Proteoglycan
NGF	Nerve Growth Factor
NgR	Nogo Receptor
NMDA	N-Methyl-D-Aspartate
NO	Nitric Oxide
NPC	Neural Progenitor Cell
N.S.	Not Significant
NSCISC	National Spinal Cord Injury Statistical Center
NSPC	Neural Stem / Progenitor Cell
NT	Neurotrophin
O4	Oligodendrocyte antigen O4
OEC	Olfactory Ensheathing Cell
Olig 1/2	Oligodendrocyte Transcription Factor 1/2
OMgp	Oligodendrocyte Myelin Glycoprotein
OPC	Oligodendrocyte Precursor
PBS	Phosphate Buffered Saline
PD	Proton-Density
PDGF-AA	Platelet Derived Growth Factor-AA
PFA	Paraformaldehyde

List of Abbreviations

PLP	proteolipid protein
PNS	Peripheral Nervous System
Prediff.	Predifferentiated
PSA-NCAM	Poly-Sialated Neural Cell Adhesion Molecule
RAG	Regeneration Associated Gene
Rb	Rabbit
RF	Radio Frequency
RhoA	Ras homolog gene family, member A
Rhox	Rhodamine X
RIP	Receptor Interacting Protein
RO	Rostral
Rt	Rat
RT-PCR	Real-Time Polymerase Chain Reaction
SC	Spinal Cord
SCI	Spinal Cord Injury
SD	Standard Deviation
SE	Spine Echo
SEM	Standard Error of the Mean
Sema	Semaphorin
SNR	Signal-to-Noise
SPRR	Small Proline-rich Repeat Protein
SVZ	Subventricular Zone
T	Tesla
TBS	Tris-Buffered Saline
TE	Echo Time
TGF- β	Transforming Growth Factor beta
TNF-alpha	Tumor Necrosis Factor-alpha
TR	Repetition Time
TSE	Turbo Spin Echo
VEGF	Vascular Endothelial Growth Factor
VSVG	Vesicular Stomatitis Virus G
WM	White Matter

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